Assessing Human Fecal Contamination in a Mixed-Use Watershed Using Microbial Source Tracking

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DEDICATION

To those who matter most: Mom, Dad, Shoshanna, and Andrew

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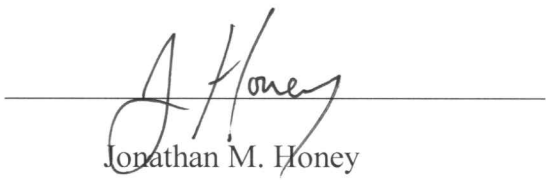

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ABSTRACT

Assessing Human Fecal Contamination in a Mixed-Use Watershed Using Microbial Source Tracking

Jonathan M. Honey, Master of Science in Public Health, 2017

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Protecting surface waters from fecal pollution is critical to protecting public health. Human fecal contamination, in particular, poses a significant risk to human health because it contains an abundance of human pathogens. While routine monitoring of standard fecal indicator bacteria (FIB), such as E. coli, has significantly reduced public exposure to pathogens, standard FIB do not enable determination of the host-species from which the fecal matter originates. Identification of host-species enables water quality managers to implement the most efficient and effective mitigation strategies. Rock Creek has, for many years, been designated as an "impaired" waterway due to fecal contamination.

The primary objective of this study was to determine the current proportion of human-associated FIB in Rock Creek. To meet this objective, quantitative polymerase chain reaction (qPCR) was employed to determine the proportion of human Bacteroides

present in Rock Creek. Water samples were collected and standard water quality parameters, including dissolved oxygen, total dissolved solids, turbidity, pH, temperature, and E. coli, were monitored over a ten-week period. Precipitation, hydrographic, and land-use data were collected to assess their impact on water quality parameters.

Data analysis revealed the following: E. coli and water temperature exceeded regulatory standards; the mean proportion of human Bacteroides was 57% (CI: 40-74%, n=5); there was a moderate, positive correlation between rainfall and [E. coli] (r=0.545, p=.011, n=21); there was a moderate, positive correlation between [E. coli] and human Bacteroides proportion (r=0.404, n=5, p=0.501); and there was no significant difference across land-use types and [E. coli] (p-0.142, n=20).

Rock Creek remains impaired due to elevated temperatures and standard FIB, with humans potentially being a significant contributor to the fecal load, although this conclusion must be regarding with extreme caution owing to numerous study limitations.

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STATEMENT OF PURPOSE

The primary purpose of this study was to determine the proportion of human-associated fecal indicator bacteria (FIB) present in the Maryland portion of Rock Creek (RC). Secondarily, this study sought to determine whether correlations exist between human fecal levels in Rock Creek and a number of variables, including significant rain events, land-use type, nearby sewer system attributes, and Escherichia coli (E. coli) levels. Microbial source tracking (MST), using real-time Polymerase Chain Reaction (PCR) analysis to determine the proportion of human fecal bacteria, was employed to achieve the primary objective.

BACKGROUND AND SIGNIFICANCE

Rock Creek is designated by the Montgomery County Department of Environmental Protection as an impaired waterway due to failure to meet several water quality criteria, one of which is elevated levels of FIB (3). A 2001 microbial source tracking study in RC concluded human feces contributed 10.5% of the fecal load in the creek (14). Fecal contamination, especially from human sources, poses a very serious public health, environmental, and economic concern.

Over the past decade, remediation efforts have been implemented to improve the water quality in RC. One ongoing measure is the upgrading of sanitary sewer lines running adjacent to the creek. The findings of this study will offer insight on the effectiveness of previously employed water contamination mitigation strategies and will inform policy makers and public health officials so that future efforts can be more efficiently directed.

Surface Water Quality and Protection

Surface water has been defined by the United States Environmental Protection Agency (U.S. EPA) as "all water naturally open to the atmosphere (rivers, lakes, reservoirs, streams, impoundments, seas, estuaries, etc) and all springs, wells, or other collectors which are directly influenced by surface water" (59). In the United States (U.S.), the overarching federal legislation which protects the nation's navigable waters is the Clean Water Act (CWA) (11). This statute is administered by the U.S. EPA and its' regulations are codified in the Code of Federal Regulations (CFR) (Title 40, Chapter 1). Under this legislation, states are obliged to provide biennial reports to the U.S. EPA on the quality of their rivers, lakes, and estuaries. Each body of water is designated for one or more "designated uses" (see Table 1), each of which has specific water quality standards (WQS) that must be achieved. The "Use Class" is a set of "designated uses that apply to a water body which individually may or may not be supported now, but should be attainable" (15). The state itself can set their own specific WQS, so long as they are scientifically defensible and protective of the designated use criteria (56). If the body of water fails to meet one or more of the criteria for its designated uses, it is deemed to be "impaired" (26). If impaired, the state must develop Total Maximum Daily Limits (TMDLs) for that particular body of water (56). A TMDL is a calculated estimate of the amount of pollutant a body of water can receive in order to maintain or achieve its designated use criteria (11). The TMDL estimate is based on the sum of the waste-load allocation (point source pollutants), load allocation (non-point source pollutants), and margin of safety (46).

Table 1. Designated Use Classes for Maryland's Surface Waters

Use Class	Description
I	Water Contact Recreation, and Protection of Non-tidal Warm-water Aquatic Life
I-P	Water Contact Recreation, Protection of Aquatic Life, and Public Water Supply
II	Support of Estuarine and Marine Aquatic Life and Shellfish Harvesting
II-P	Tidal Fresh Water Estuary – includes applicable Use II and Public Water Supply
III	Non-tidal Cold Water
III-P	Non-tidal Cold Water and Public Water Supply
IV	Recreational Trout Waters
IV-P	Recreational Trout Waters and Public Water Supply

Reference: Department of the Environment (Maryland) (15)

Point source pollution is defined by the U.S. EPA (55) as "any discernable, confined and discrete conveyance, including but not limited to any pipe, ditch or concentrated animal feeding operation from which pollutants are or may be discharged". It includes discharges from stormwater drains, inefficient sewage treatment plants, combined sewer overflows (CSOs), and industrial sources. Non-point sources include leaking septic systems, wildlife, and run-off from agriculture, forestry, and urban sources (2; 42). Upon establishing a TMDL, pollutant dischargers must apply for a permit under the National Pollutant Discharge Elimination System (NPDES), a program that requires dischargers to meet specific effluent limits and monitoring (55).

There is a highly complex interaction of a multitude of factors, both anthropogenic and environmental, which can impact surface water quality (Figure 1). Water pollutants include pathogens, oxygen-demanding wastes, nutrients, salts, thermal pollution, heavy metals, pesticides, volatile organic compounds, and other emerging contaminants (e.g. pharmaceuticals, detergents, and nano-particles) (26). It is beyond the scope of this study to discuss each of these factors; however, those that will be examined

in the current study (precipitation events and sewer systems) are described in greater detail below.

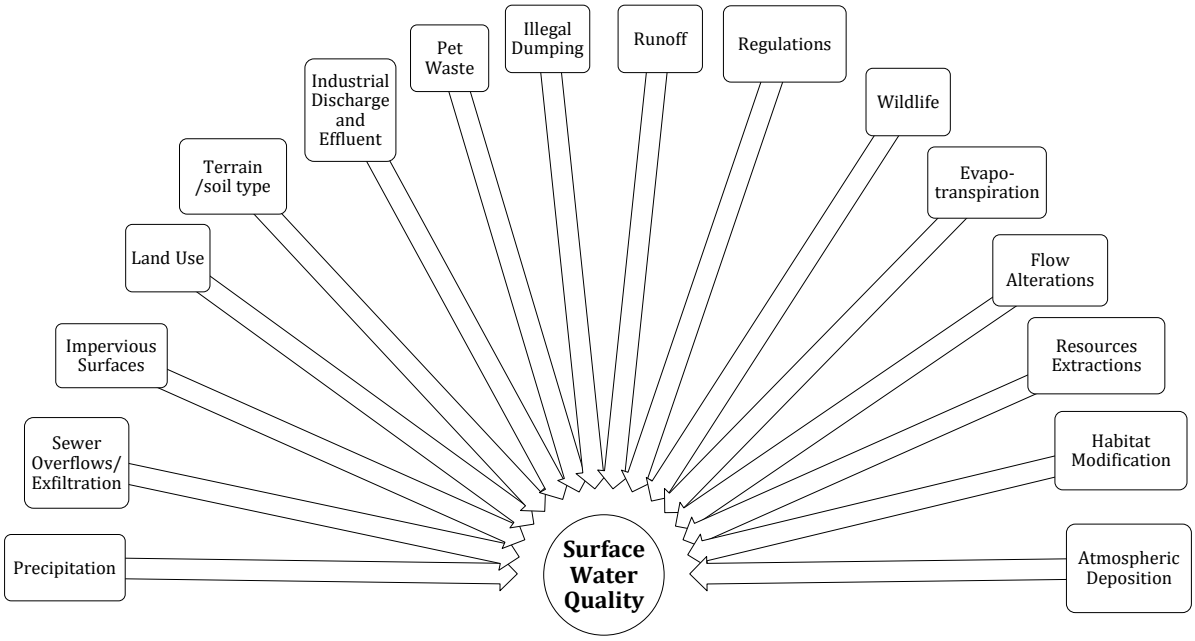

Figure 1. Factors Influencing Surface Water Quality
Reference: Masters and Ela (26)

Precipitation Events and Runoff

Rainwater itself is not pure (i.e. hydrogen and oxygen alone), especially in developed areas where it absorbs many atmospheric pollutants (e.g. gases and dusts) as it falls to earth. Upon reaching the ground, rainwater can dislodge soil particles (or the materials, such as pathogens that are sorbed to soil (42)), dissolve mineral and organic matter from the earth, and transport many anthropogenic pollutants (e.g. fertilizers, motor oil, pet waste, and agricultural waste) that contaminate the ground (Figure 2) (5). Without mitigation methods in place, runoff can carry these pollutants directly into surface waters (7).

Urban runoff consists of dry-weather base-flow, snow-melt, CSOs, and stormwater runoff. Agricultural and urban runoff, the major sources of which are "construction sites, on-site sewage disposal systems, households, roadways, golf courses, parks, service stations, and parking areas" (32), together contribute the largest sources of stormwater pollutants in streams and rivers (7). Of particular importance to the current study, sources of pathogen-contamination in stormwater include "farm runoff, landfill and lagoon runoff and leachate, and on-site septic system (leachfield) overflows" (7).

With significant rain events, there is an associated increase in runoff and, commonly, a concomitant increase in pollutants carried to surface waters. Significant rain events have been closely associated with fecal contamination of waterways (42). Stormwater runoff, particularly during warmer weather, commonly contains high levels of fecal bacteria (7).

Heavy precipitation can also lead to CSOs (described in greater detail below), as a rapid influx of stormwater into the sewer system may exceed the capacity of the combined sewer pipe itself or the capacity of the wastewater treatment facility (22). Interestingly, in such situations where heavy rainfall leads to increased run-off, it is possible, although rare, to see a decreased proportion of human fecal contamination due to the effect of dilution (36).

Runoff is also increased in areas with more impervious surfaces (e.g. rooftops, roads, and parking lots) as infiltration into the ground is decreased. In bypassing soil absorption, bacteria found within runoff are not exposed to the mechanisms (i.e. sedimentation, sorption, and inactivation) that could otherwise remove bacteria from runoff (7). When stormwater (from agricultural and urban runoff) is directed to

waterways that are used for fishing, swimming, or drinking water supplies, the significance of the pollutants (especially toxicants and pathogens) becomes much more critical from a public health perspective (7).

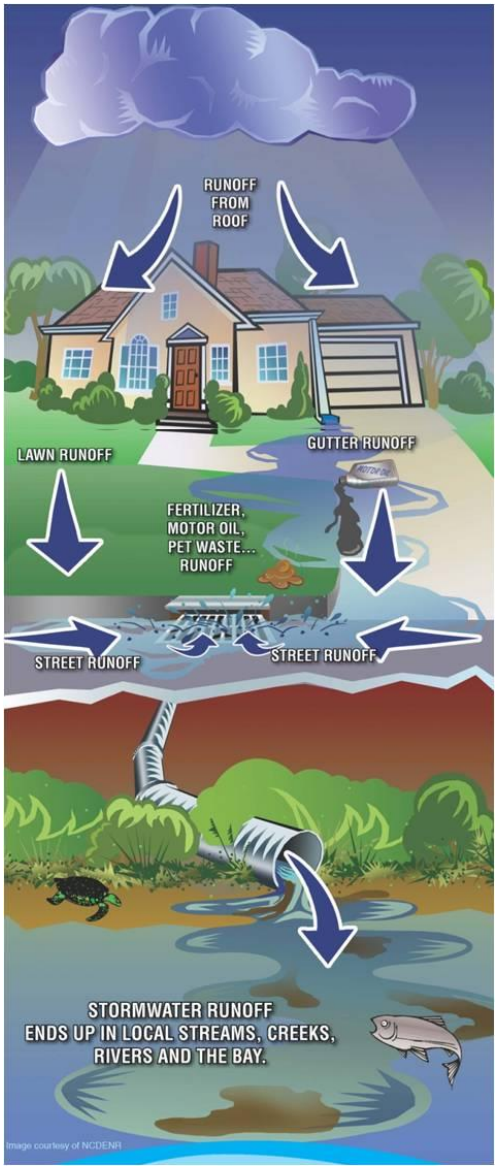

Figure 2. Impervious Surfaces and Runoff Contribution to Surface Water Pollution Credit: North Carolina Department of Environmental Quality (NCDEQ) (30)

Sewer Overflows and Exfiltration

The three main categories of sewers include sanitary sewers, which carry sewage and industrial wastewater from "residences, commercial buildings, industrial plants and institutions"; storm sewers, which are intended to "carry only storm waters, surface run-off, street wash waters, and drainage"; and combined sewers, which are intended to carry both sanitary sewage or industrial wastewater and storm water (1).

Many sewer systems in the U.S. are deteriorating because they have been in place, and not well maintained, for many decades. This deterioration can result in exfiltration, which is the escape of wastewater from the sewer system, leading to contamination of nearby groundwater, surface waters, and storm sewers (1).

It is important to distinguish between sanitary sewer overflows (SSOs), combined sewer overflows (CSOs), and exfiltration. SSOs are the result of infiltration and inflow which results in excessive volumes of wastewater in the system, which can lead to "overflows to receiving water, street flooding, and basement flooding" (1). Combined sewer overflows (CSOs) typically occur during heavy rain events, when urban runoff and sanitary sewage is combined, and the capacity of the sewage system (piping or wastewater treatment facility) is exceeded. A CSO event can lead to raw (untreated) sewage being released directly into surface waters (61). Because combined sewers are commonly older and more shallow than separate sewers and constructed with less-watertight fittings, they are more likely to experience more significant exfiltration events (1). Replacing combined sewers with separate sanitary and storm sewers is expected to reduce sewage pollution (40).

Raw sewage often contains many pollutants including, but not limited to, suspended solids, pathogens, toxicants, floatables, nutrients, oxygen-demanding organic

compounds, oil, and grease (1). The release of raw sewage into surface waters will negatively impact the water quality and can pose serious public health threats, compromise aquatic life, and reduce the recreational usability of the waterways (1).

Sewer overflows, as compared to exfiltration events, are presumed to be much more likely to contaminate surface waters. This is due to the fact that sewers that are located near surface waters are typically below the groundwater level and are therefore more likely to experience infiltration (as opposed to exfiltration) events. In some scenarios, however, where the terrain near a body of water is very steep, it could occur that a sewer pipe runs above the ground water level and would therefore be susceptible to exfiltration (1). Additionally, a recent study by Sercu et al (40), demonstrated evidence that exfiltration of sanitary sewers, running above storm sewer pipes, can lead to contamination of the storm sewers and subsequent surface water contamination.

Fecal Contamination

In 2005, the U.S. EPA reported that 13% of surface waters in the U.S. failed to meet designated use criteria due to elevated levels of FIB. Despite the significant reductions in point-source water contamination following the 1972 implementation of the NPDES, fecal contamination remains a significant threat to U.S. waters (44; 55). The threat continues, as confirmed recently by the U.S. EPA, that 39.2% of all U.S. rivers, lakes, and streams are unsafe for recreational use, primarily due to fecal contamination (45). Fecally polluted waters pose a significant human health threat and can lead to deleterious environmental and economic effects (39). Human fecal pollution, in particular, because of its abundant quantity of pathogens, has been identified as posing a

higher human health risk (19). For that reason, and so mitigation efforts can be more focused, it is prudent to identify the source of fecal contamination (28).

Fecal pollution of surface waters persists, in part, due to the inherent difficulties of determining the origin of non-point source contaminants. Recent reports confirm that fecal microbes are the most common biological contaminant in U.S. waters (58). Microbial source tracking is an emerging method that may be helpful in identifying non-point sources of fecal contamination (48).

Fecal matter from warm-blooded animals contain numerous types of microorganisms (viruses, bacteria, and protozoa). Many of these organisms (e.g. Salmonella, Shigella, and Vibrio) are pathogenic to humans, meaning they can be disease causing when in contact with or ingested by humans (29). There are numerous reasons why it is not practicable to attempt to monitor every specific human pathogen. The analytic techniques are often time consuming, expensive, and require highly trained experts; it would be nearly impossible to determine which of the various pathogens to target; and the pathogens may have very short survival times outside of the host or may be present in vary low concentrations and therefore difficult to detect (21; 22). FIB are particular organisms selected to assess the microbiological quality of a body of water. An ideal FIB has specific properties; such as being easily detected, of human/animal origin, surviving at least as long as the pathogens, present at densities correlated with fecal contamination, a surrogate for many different pathogens, and appropriate for fresh and/or marine waters. While FIB are typically not pathogenic to humans, their presence indicates that of fecal matter, which likely contains human pathogens (29). The benefit of monitoring FIB as a predictor of microbiological water quality is that it circumvents

the need to detect each of the plethora of potential pathogenic organisms that might exist in surface waters (39). The standard fecal indicator bacteria (SFIB) include E. coli, Enterococci, total coliform bacteria, fecal coliform bacteria, fecal streptococci, and Clostridium perfringens (27; 29). The U.S. EPA currently recommends enumeration of E. coli and enterococci as the FIB of choice for fresh recreational water (44; 56).

While there is substantial evidence that monitoring FIB has enhanced public health protection (50), the suitability of these SFIB as the indicators of surface water quality has been questioned for the reasons noted below (19; 22; 27; 39; 44):

1. SFIB have the ability to adsorb to soils and sediments and then, following instances such as heavy rainfall, can become dislodged and falsely indicate the presence of fecal contamination long after the contamination occurred.
2. SFIB are also able to multiply outside of the intestinal environment, leading to potentially inflated assessments of fecal contamination.
3. SFIB are found in the feces of many cold-blooded and warm-blooded animals, in addition to humans, therefore making host-origin identification difficult.
4. SFIB are found in differing numbers and ratios within the intestines of various animals and humans, making it unclear how to estimate the proportion contribution to a particular species when the contributing source is unknown.
5. There is genetic evidence suggesting that there are unique strains of E. coli and enterococci which, although assumed to have originated from fecal matter, have since evolved in non-intestinal environments (soil, sediment, and algae).

6. SFIB have been shown not to correlate well with a number of human pathogens, including Salmonella spp., Campylobacter spp., Cryptosporidium and Giardia spp., and the human enteroviruses, adenoviruses and coliphages.

Microbial source tracking has been suggested as an adjunct to SFIB assessment (22; 51).

Additional Water Quality Parameters

While it is not the emphasis of this study, there are many other parameters that provide information on the quality of surface water and are used in the assessment of designated use obtainment. Table 2 identifies many of the typical parameters used to characterize the quality of surface water. Table 3 below provides a brief description of some of the more common parameters used in characterizing surface water quality.

Table 2. Typical Factors Used in Conducting a Water Body Assessment

Physical Factors	Chemical Factors	Biological Factors
Instream characteristics - size (mean width and *depth) - flow velocity - annual hydrology - total volume - reaeration rates - gradient/pools/riffles - *temperature - sedimentation - channel modifications - channel stability Substrate composition and characteristics Channel debris Sludge deposits Riparian characteristics Downstream characteristics	*Dissolved oxygen Toxicants Suspended solids Nutrients - nitrogen - phosphorus Sediment oxygen Salinity Hardness Alkalinity *pH *Dissolved solids [*Turbidity] [*Conductivity]	Biological inventory (existing use analysis) - fish - macroinvertebrates - microinvertebrates - phytoplankton - periphyton - macrophytes Biological potential analysis - diversity indices - HIS models - tissue analysis - recovery index - intolerant species analysis - omnivore-carnivore comparison Biological potential analysis - reference reach comparison [*E. coli concentration] [*Total coliform concentration]

*Parameters measured for this study
Reference: U.S. Environmental Protection Agency (54)

Table 3. Description of Common Water Quality Parameters

Parameter	Utility
Dissolved Oxygen (DO)	DO is a necessity to support fish populations. With DO < 5 mg/L, most fish species become endangered. Organic pollution (e.g. sewage) is the main cause of decreasing DO.
Nutrients:	Nutrients (such as N and P) are necessary for aquatic plant life; however, when in excess, can lead to unwanted eutrophication (excessive algae growth) and subsequent DO depletion in surface waters (lakes and reservoirs, in particular).
- Nitrogen (N)	Nitrate (NO_3) in drinking water can pose a serious public health threat. Major source of nitrogen: sewage, animal feedlot runoff, fertilizers, and coal-fired power plants.
- Phosphorous (P)	Phosphorus is often the limiting nutrient or plant growth in rivers and lakes; in excess it often leads to unwanted algal blooms. Major sources: sewage (including detergents), and animal feedlot runoff.
pH	Extremes in pH can affect the physiological functioning of aquatic plants and animals.
Temperature	Temperature increases in surface waters usually results from the return of warmed water after it is used for cooling at power plants. Warmer water leads to DO depletion.
Total Dissolved Solids (TDS) (and Conductivity)	TDS/conductivity is a measure of salinity. Salinity levels often dictate the suitable uses for surface waters (e.g. salinity >500 mg/L is less desirable for drinking water). Industrial release of salts and agricultural irrigation are major causes of elevated salinity.
Turbidity	This is a measure of the quantity of suspended particles in water. Elevated turbidity decreases sunlight penetration thereby detrimentally effecting plant photosynthesis and aquatic animals' ability to mate and find food and shelter.

References: Masters and Ela (26), U.S. Geological Survey (57), and U.S. Environmental Protection Agency (54)

Microbial Source Tracking

Microbial source tracking is the tracing of host-specific microbes to the host-species from which they came (22). The premise of MST is that certain microbe strains are specific to a particular host-species; therefore, determining the unique genetic identity of the microbe allows for the determination of its host-species (19; 22; 25; 48).

Depending on the needs and intent of the MST study, the sources of microbial contamination can be identified with increasing granularity: from broad (e.g. human or animal) to very specific groups (e.g. by species) (64). Because fecal contamination may come from animal or human sources, point sources or non-point sources, it is important to determine the origin of contamination so as to develop the most effective mitigation strategies, including the development of TMDLs (28; 39; 51). Additionally, human fecal contamination is presumed to pose a greater human health threat than animal fecal contamination, due to its high content of pathogenic organisms (19; 22; 39). For this reason there is benefit in simply determining whether fecal contamination is of human or non-human origin (39; 51; 55).

There are two general strategies of analysis for MST: library-dependent and library-independent (Table 4). The former method identifies fecal sources based on databases or "libraries" of genotypic or phenotypic fingerprints from bacteria strains isolated from known fecal sources, whereas the latter identifies sources based on known host-specific characteristics of the bacteria or virus, without the need of a library (51).

Table 4. Common MST Methods

Library-dependent		**Library-independent**	
Culture-dependent			**Culture-independent**
Phenotypic	**Genotypic**	**Phenotypic or Genotypic**	**Genotypic**
- Antibiotic resistance - Carbon utilization	- Rep-PCR - PFGE - Ribotyping	- Bacteriophage - Bacterial culture	- Host-specific bacterial PCR - Host-specific viral PCR - *Host-specific quantitative PCR

*Technique used in the current study
PFGE = pulsed-field gel electrophoresis
Reference: Tetra Tech Inc. and Herrera Environmental Consultants (51)

Library independent methods (LIMs) typically extract deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) directly from the bacteria or viruses found in the water sample and utilize PCR to amplify and detect a source-specific genetic marker (51; 55). LIMs have gained preference in recent years as the improved techniques and equipment have made this method easier, faster, and less expensive (51). A comparison of the advantages and disadvantages of various MST methods is provided in Appendix B.

Other (non-microbial) source-tracking methods do exist and include detection of human-associated fecal chemicals (e.g. caffeine, fragrances, and detergents) and particular fecal constituents (e.g. sterols, stanols, and immunoglobulins) (55).

Two key components of the MST method are the selection of the tracer microbe and the analytic method of determining its identity. A tracer microbe is used for the same reasons stated previously for utilizing FIB to indicate the presence of fecal contamination (i.e. a tracer is a more convenient, realistic, time-saving surrogate for the multitude of other potential pathogens that could be present in a water sample). The ideal tracer is said to have the following characteristics (19; 39):

- presence should correlate with that of the pathogens of interest
- a survival profile similar to that of the pathogens
- not reproduce outside of the host
- non-pathogenic
- rapidly detectable
- easily enumerated

A number of studies have suggested that Bacteroides may be a suitable FIB for MST, as they make up a significant portion of fecal bacteria in humans and animals, are

relatively persistent in the environment, do not grow in the environment, are host-specific, and can be analyzed quite rapidly (19; 25; 27). Bacteroides are a genus of gram-negative, obligate anaerobic (fecal) bacteria. Because Bacteroides are anaerobic, it is challenging and time-consuming to make cultures; however, human and animal host-specific genetic markers have been elucidated, therefore enabling DNA analysis via PCR techniques, which do not require culturing (51). Specifically, numerous studies have focused on and developed primers (e.g. HF183) to target particular sequences of the 16S rRNA gene (22).

There are various analytic methods for determining the identity and quantity of the selected FIB, one of the emerging favourites being the quantitative PCR (qPCR) (43; 44; 48). Quantitative PCR enables researchers not only to determine the presence or absence of a microbial DNA in a water sample, but also allows quantification of the microbial DNA, thus informing us of the proportion of fecal contamination contributed by the various sources (51).

Polymerase Chain Reaction

PCR is a molecular method that allows for a selected sequence of DNA to be amplified, resulting in millions of copies of DNA, which can then be visualized and/or quantified (6). A recently developed and more accurate quantification technique is termed real-time PCR, in which "real-time" indicates that the synthesis of product DNA is measured throughout the PCR cycles. Real-time PCR and qPCR are generally used synonymously in the scientific literature today. The process for qPCR is the same as for conventional PCR, except that a fluorescent DNA-binding dye (e.g. SYBR® Green I) is added to the initial mixture. This allows for real-time measurements of fluorescent

signaling of the product DNA and comparison with the control PCR to determine the quantity of product DNA present (6; 37). The usage of qPCR in water quality testing, and MST in particular, has steadily increased over the past two decades. In 2005, the U.S. EPA published a comprehensive MST Guide Document which included the application of culture-independent, library-independent approaches (of which PCR is the primary method). At that time, because there was insufficient evidence, the U.S. EPA could not recommend this method over any others (55). However, as of 2014, the U.S. EPA favours qPCR-based DNA amplification testing as the most ideal MST approach and is spearheading a major research effort to standardize the qPCR methods (43).

The utility of qPCR in MST studies can be seen in the case of a fecally polluted water source. For instance, discriminating between human and bovine fecal contamination in a waterway might influence whether mitigation strategies are directed toward sewer repairs or reduction of agricultural run-off. If researchers and water quality managers are able to rapidly determine the origin (and quantity) of fecal matter contamination based solely on the presence (and quantity) of specific microbial DNA, then mitigation strategies can be more targeted, timely, and effective.

Study Area

The Rock Creek watershed covers approximately 197 km^2, with approximately 80% of the drainage within Montgomery County, Maryland (MD) (Figure 3). The mainstream of RC originates in Laytonsville, MD and flows 37 km south through Montgomery County and continues for another 15 km within the District of Columbia (D.C.) where it discharges into the Potomac River and eventually the Chesapeake Bay. North of Maryland Route 28/Norbeck Drive, Rock Creek is referred to as Upper Rock

Creek and south of this landmark, it is referred to as Lower Rock Creek. For the purpose of this study, the portion of Lower Rock Creek that flows through Maryland (excluding that which runs through D.C.), will be referred to as Maryland's Lower Rock Creek (MDLRC) and defined as that portion of the creek which runs south of MD Route 28/Norbeck Drive to the D.C. border. Most of MDLRC runs through a densely populated and developed area, making it susceptible to urban run-off pollution (3; 18).

The Upper Rock Creek watershed, which is north of Fieldcrest Road, is relatively undeveloped and is protected to some degree by "stream valley parkland buffers" (13). According to the Department of Environmental Protection (Maryland):

> Land uses in the drainage area from Fieldcrest Rd. downstream to Muncaster Rd. consist of newly developing large-lot residential subdivisions, commercial lots along Route 124, and existing low- to medium-density residences. Between Muncaster Rd. and Muncaster Mill Rd., Rock Creek increases in size as its drainage area enlarges. Medium-density residential development predominates, although there are still areas of large-lot developments in the drainage. The stream valley in this area is in succession from farm fields to young forest (13).

Rock Creek Regional Park is located within the Upper Rock Creek Watershed. The park contains two man-made lakes, Lake Needwood (~ 0.3 km^2 or 75 acres) and Lake Frank (0.22 km^2 or 55 acres). These lakes were constructed in the 1960s for the purposes of water quality control, flood-control, and recreation (34; 35).

Within the Upper RC watershed, there are Special Protection Areas (SPAs), the designation of which "requires use of enhanced plan review, sediment and erosion

control, and stormwater management techniques for new development in order to provide additional stream resource and water quality protection" (3). These SPAs include the main stream north of Muncaster Mill Road and the western side of the North Branch of Rock Creek north of Muncaster Mill Road (3).

The MDLRC watershed has seen a steady increase in development and population and is currently "heavily urbanized and densely populated" (13). Within this watershed is Rock Creek Park, which is contiguous with the Rock Creek National Park in D.C. Rock Creek Park is not only an attraction for recreational activities (hiking, running, cycling, etc.), but it also serves as a protective buffer along the creek, "preserving vernal pools and wetlands in the floodplain" (13).

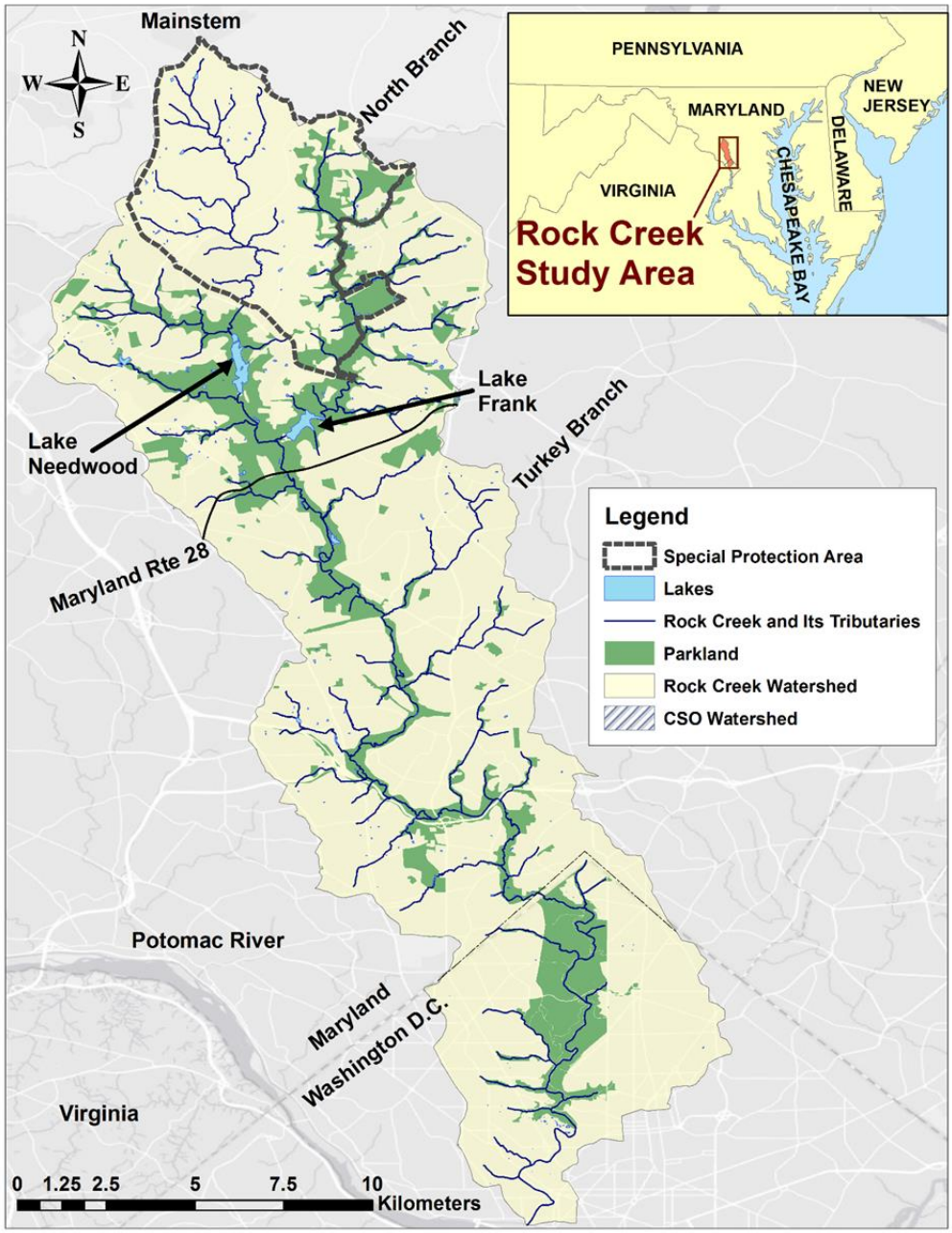

Figure 3. Rock Creek Watershed
Credit: Cintron (10)

The "designated use classes" of Rock Creek are illustrated in Figure 4 and include:

1. Use Class III (non-tidal cold water): From the headwater in Laytonsville to the north junction of Lake Needwood
2. Use Class IV (recreational trout waters): From the south tip of Lake Needwood to just south of Norbeck Drive

3. Use Class I (water contact recreation and protection of non-tidal warm-water aquatic life): From just south of Norbeck Drive to the D.C. border (15)

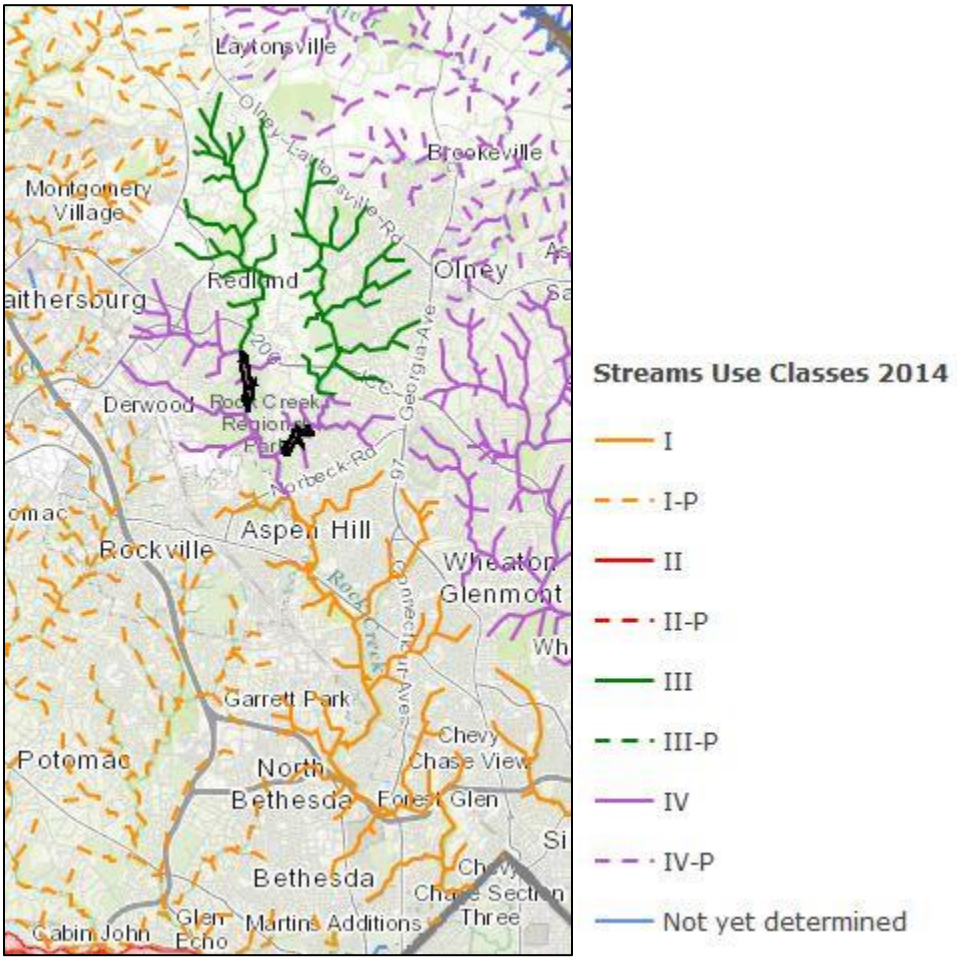

Figure 4. Designated Use Classes for Maryland's Surface Waters
Credit: Department of the Environment (Maryland) (15)

Land Use and Impervious Surfaces

Land use types, ranging from forest, agricultural, to urban, have been shown to have substantial impact on nearby watersheds. Changes in land use have been found to be associated with alterations in run-off, generation of non-point source pollution, and influencing surface water quality (63). The land use types affecting the Rock Creek watershed includes low- and medium-density residential and recreational land, with less

than seven percent of the land-use is identified as forest, open water, or bare ground. Sixty-five percent of the land-use is deemed residential, followed by 10% municipal/institutional, and 8% roadways (3; 10). As discussed previously, runoff quantity is increased by impervious surfaces. Impervious surfaces make up approximately 21% the Rock Creek watershed. The SPAs noted above are regulated to allow no more than eight percent new impervious surfaces (3).

Sewer Service

Two public sanitary sewer lines run adjacent to the entire length of the Montgomery County section of Lower RC and are located, with few exceptions, within 30 meters of the creek (14). These gravity sewer lines, operated and maintained by the Washington Suburban Sanitary Commission (WSSC), flow south to the Blue Plains Wastewater Treatment Facility, located in Washington, D.C. (3). These sewer lines were installed over fifty years ago and, as part of a multi-year upgrade project which began in 2005, continue to undergo the much needed replacement or repair (3; 62).

Existing Water Quality in Rock Creek

One method used to assess the quality of a body of water is to measure the health of the aquatic biological community. Fish and arthropods can thrive in healthy, clean waters, but their populations will decline in polluted waters. As depicted in Figure 5, the health of Upper Rock Creek is relatively unimpaired and its condition is rated as good, while the conditions in MDLRC are rated as poor to fair (13). The Stream Condition Index is based upon the Index of Biotic Integrity (IBI), which is a multi-metric score which reflects the number and type of fish and bugs living in the body water (Table 5) (13).

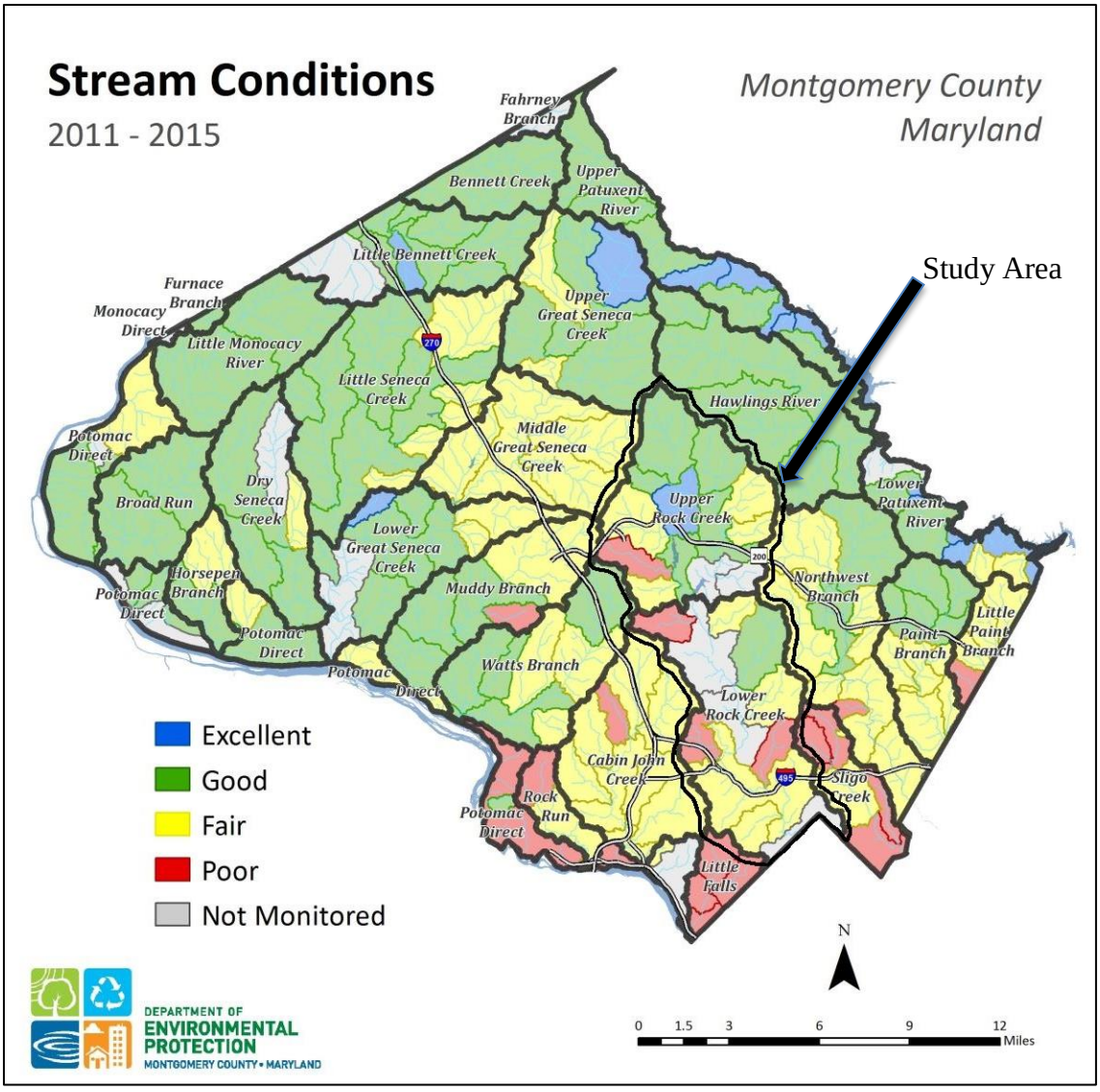

Figure 5. Stream Conditions, Montgomery County, Maryland
Credit: Department of Environmental Protection (Maryland) (13)

Table 5. Stream Condition Index and Index of Biotic Integrity

Stream Condition Index	IBI Score (explanation)
Poor	0-41 (Poor conditions most often occur in places where changes made by humans to the natural environment have substantially altered the structure of the biological community. These areas are often highly developed or urban and don't have good stormwater management)
Fair	42-63 (These conditions occur most often in places anthropogenic stressors have impacted an area, but the area still supports viable biological communities. This condition describes many streams in suburban areas with some stormwater management, as well as areas that have had major agricultural impacts. The biological communities in fair streams are dominated by species that are tough and can survive in most conditions, but may have a few organisms that are sensitive to stressors left)
Good	64-88 (These conditions are often found in the less developed areas of the county, suburban areas with the latest stormwater management techniques, and areas with lots of protected land in their watershed. Many of the County's sensitive species can survive in these streams. Stream bugs like dragonflies and caddisflies are common. Fish like sculpins, darters, and longnose dace are common in these streams as well)
Excellent	89-100 (Most often, only highly forested watersheds with minimal development are in excellent condition. Here our most sensitive fish and stream bugs live. Fish like trout, shield darters, and comely shiners are found. Highly sensitive stream bugs like stoneflies and mayflies are common in these watersheds)

Reference: Department of Environmental Protection (Maryland) (13)

According to a Biohabitats Inc (3) report prepared for the Montgomery County Department of Environmental Protection, impairment designation in RC is based on findings of elevated phosphorus, total suspended solids, fecal bacteria, and impacts to biological communities. Approximately 44% of RC was rated as having poor to very poor biological conditions. Anthropogenic sources of phosphorus include fertilizers, chemicals, animal waste, and municipal sewage. High conductivity, also identified as a parameter causing RC to be designated as impaired, was linked to urban run-off, road salts, fertilizers, and leaking sewers. The report recommended additional water chemistry

analysis and monitoring of phosphorus, conductivity, and related parameters. Phosphorus and conductivity are indicators of unwanted conditions, such as eutrophication and presence of toxic inorganic chemicals (3).

The only known microbial source tracking (MST) study in Lower RC, conducted by the Department of Environmental Protection (Montgomery County) (14), reported fecal sources as 20.3% avian, 19% pets, 11.9% wildlife, 10.5% human, 3.7% agriculture, and 34.6% unknown (Figure 6). That study indicated the fecal contamination of Lower RC had no impact on the D.C. portion of the Creek. Also, it concluded there was no evidence to suggest that any particular species of local wildlife required reduction; instead, they recommended an emphasis on the reduction of contamination by human and pet waste. A 2012 report stated that pet waste management in the vicinity of Rock Creek would have the largest impact on bacterial loads for the least cost expenditure (3).

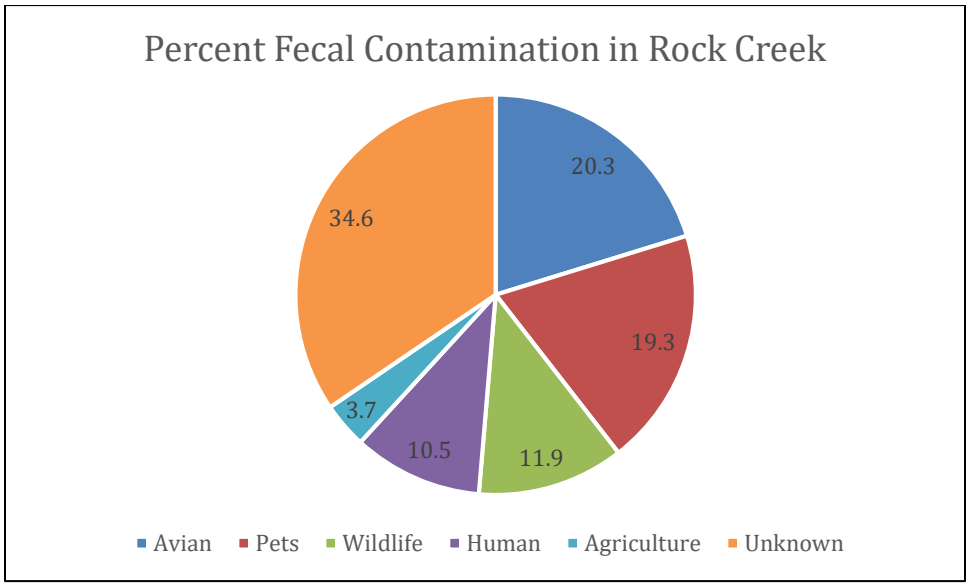

Figure 6. Percent Fecal Contamination in Rock Creek
Reference: Department of Environmental Protection (Montgomery County) (14)

A study conducted by Uniformed Services University of the Health Sciences (USUHS) graduate student, Nicole Cintron (10), involved collecting water samples weekly from 15 sites along the entire length of Rock Creek between July and October 2015. Samples were analyzed for turbidity, nitrogen, phosphorus, *E. coli*, and total coliforms. The purpose of that study was to investigate environmental and anthropogenic factors (such as precipitation, temperature, sewer characteristics, impervious surfaces, and land use) that impact the surface water quality in the Rock Creek watershed. Of relevance to the current study, a positive (although not statistically significant) correlation was found between significant rain events, temperature, and discharge and mean enteric bacteria concentration. A "significant rain event" is defined by Cintron (10), through correspondence with the Montgomery County Department of Environmental Protection, as "one resulting in accumulated rainfall greater than one half of an inch over a 24-hour period". One of the recommendations made by Cintron was to conduct an MST study in Rock Creek to determine the distribution of host-specific fecal contamination, to assist in identifying the sources (i.e. point or non-point sources) of contamination.

Public Health Relevance

Clean and safe water sources are crucial to human health. Whether used for drinking water, recreation, or fishing, polluting these water systems can lead to devastating health and economic effects (such as closures of beaches or shellfish harvesting areas). Waters contaminated with human feces (versus animal feces) are generally considered to pose a more significant threat to human health, as they are likely to contain a multitude of human pathogenic organisms, such as salmonella, shigella, hepatitis A virus, and norovirus (19; 22; 39). Because qPCR-based MST can provide

water quality results relative rapidly (within hours), it offers the advantage over culture methods, such as that currently recommended by the U.S. EPA, which can take a full day to obtain results on the quality of the water. This delay in information can lead to an increased risk or unnecessary closure of waterways (47).

The parklands surrounding much of Rock Creek are an important natural resource, serving as habitat to a variety of wildlife and plants and providing a multitude of recreational activities. Pollution, including fecal contamination, threatens many of these attributes (33). As depicted in the exposure pathway below (Figure 7), by determining whether fecal contamination in Rock Creek surface water is from human or animals (and the proportion of each), previously employed mitigation strategies can be assessed and future efforts can be more efficiently directed. Determining the source of fecal pollution can lead to improved water quality management (43) and the protection of human health during recreational activities.

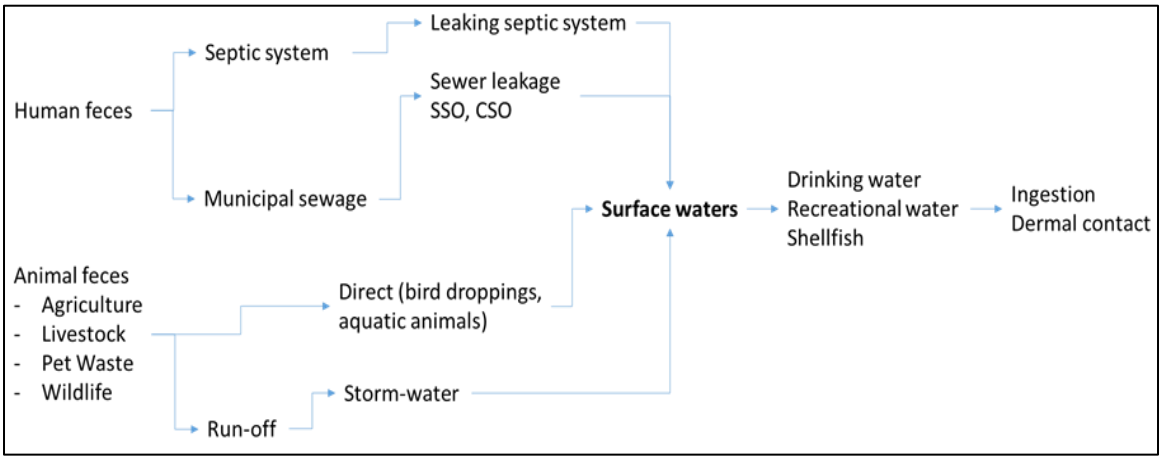

Figure 7. Exposure Pathway, Fecal Source to Human Ingestion/Contact

The use of qPCR in MST and water quality management is still a relatively new area of study. In particular, standardized methods for identifying human-associated fecal content in public waterways are currently being extensively studied and refined (43).

This study will employ current techniques to contribute to the knowledge base of how best to utilize this promising method in improving water quality and protecting public health.

STUDY OBJECTIVES

The primary objective of this study was to determine whether the proportion of human-associated fecal contamination in RC less than or equal to the last known measure of 10.5%.

The secondary objectives of this study were to determine whether:

1. There a correlation between the quantity of E. coli detected via traditional methods and the proportion of human Bacteroides genetic markers detected via qPCR in RC.
2. Significant rain events lead to an increased proportion of E. coli and/or human-associated fecal contamination in RC.
3. A relationship exists between influenced land-use type and E. coli and/or human-associated fecal contamination levels in RC.
4. Older (unrepaired) sections of the main sanitary sewer lines adjacent to RC correlate with an increased proportion of human-associated fecal content in RC.

CHAPTER 2: MATERIALS AND METHODS

To meet the stated objectives of this study, real-time water quality measurements were taken in RC and water samples were collected from RC for lab analyses to determine the turbidity, FIB concentrations, and the proportion of human fecal contaminants present. Statistical analysis was conducted in order to address this study's research questions. The following sections provide a more detailed description of the methods that were used.

Site Selection

Six sites were selected for sampling (Table 6). Five sites (A, B, C, D, and F) were selected from a pool of sites used in a recent study of the Rock Creek Watershed by Cintron (10). The sixth site (Site E) was selected to fill the geographic gap not covered in the Cintron study. Site E coincides with a sampling site used in the MST RC study by the Department of Environmental Protection (Montgomery County) (14). While Sites A, B, C, D, and F are the same as those used by Cintron, the naming system is different (Table 6). Selection determination incorporated influenced land-use, as determined by Cintron, main sewer line age, distribution of sites along the length of RC, and proximity to a US Geological Survey (USGS) Gauge Station (Site C). A map of the study area is provided below (Figure 8). Authorization to perform water sampling in RC was obtained from the Montgomery County Department of Parks, Maryland-National Capital Park and Planning Commission (Appendix B).

Table 6. Sample Site Locations

Site	Description	Latitude (N)	Longitude (E)	Influenced Land-Use	Corresponding Site Names from Cintron (10)
A	Northernmost point, within Agricultural Farm Park	39.160556	-77.131111	Low-density residential	A
B	South of Baltimore Rd Bridge	39.090000	-77.115278	Recreational	D
C*	On Turkey Branch, along Matthew-Henson Trail	39.068333	-77.081389	Recreational	F
D	South of confluence with Turkey Branch at Winding Creek Park	39.057949	-77.093088	Recreational	G
E	South of the confluence of Joseph's Branch and Rock Creek	39.035667	-77.085032	Medium-density residential	--
F	South of E-W Highway and confluence with Rock Creek	38.991694	-77.061590	Medium-density residential	H

* Site C is located on Turkey Branch (at USGS Gauge Station) not on Rock Creek

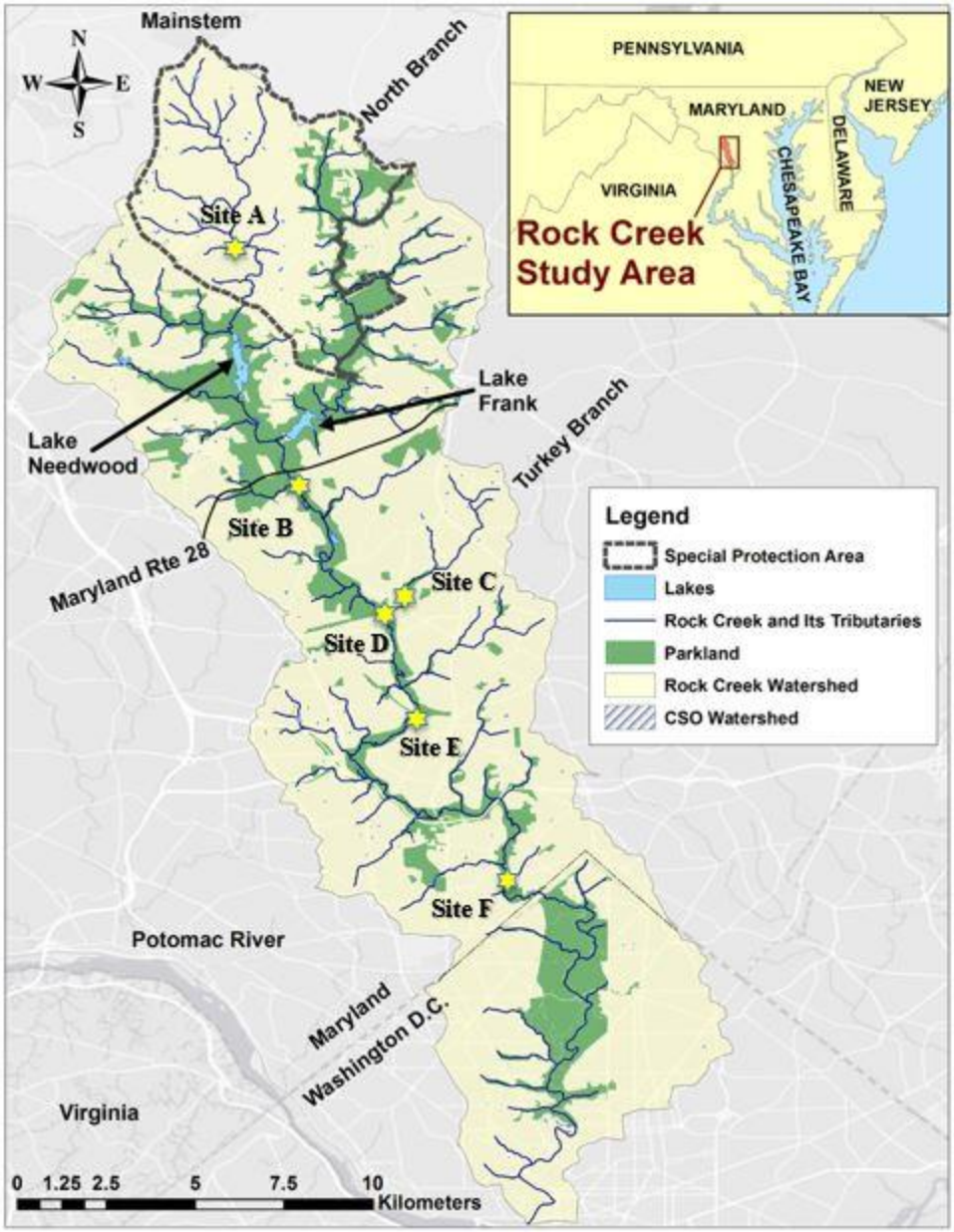

Figure 8. Sample Site Locations
Credit: Cintron (10)

Sampling Schedule

Samples were collected on various weekday mornings from 20 July to 20 September 2016, plus an additional sampling day on 7 February 2017, as shown in Table 7. For consistency and convenience, sites were sampled in order from south to north

(Sites F through A). Samples were collected from each of the six sampling sites on each sampling day.

Table 7. Sampling Dates

Sampling Event	Date	Sampling Week	Day of the Week
1*	20-Jul-16	1	Wednesday
2	22-Jul-16		Friday
3	25-Jul-16	2	Monday
4	27-Jul-16		Wednesday
5	29-Jul-16		Friday
6	1-Aug-16	3	Monday
7	3-Aug-16		Wednesday
8	5-Aug-16		Friday
9	8-Aug-16	4	Monday
10	10-Aug-16		Wednesday
11	12-Aug-16		Friday
12	15-Aug-16	5	Monday
13	17-Aug-16		Wednesday
14	19-Aug-16		Friday
15	23-Aug-16	6	Tuesday
16	30-Aug-16	7	Tuesday
17*	2-Sep-16		Friday
18	6-Sep-16	8	Tuesday
19	9-Sep-16		Friday
20	13-Sep-16	9	Tuesday
21	20-Sep-16	10	Tuesday
22**	7-Feb-17	11	Tuesday

*Quality control - field and trip blanks analyzed
**Additional sampling day

Field Data Collection

To collect water samples, entry into the creek occurred at least one meter downstream of the sampling point, so as not to disturb the creek bottom or introduce contaminants upstream of the sampling point. The sampler wore clean elbow-length, re-useable nitrile gloves throughout sampling. The nitrile gloves were pre-washed with

soap and water and dried at least one day prior to sampling. The gloves were triple rinsed in the creek, downstream of the sampling point, prior to collecting each water sample.

A calibrated portable PC450 meter (Oakton Instruments, Vernon Hills, IL, USA) was used to obtain real-time measurements of conductivity, pH, water temperature, and total dissolved solids at each sampling point. A calibrated portable meter (Hach HQ30d, Loveland, CO, USA) was used to obtain a real-time dissolved oxygen measurement at each sampling point. These data were stored in the respective meters and immediately entered into a field notebook. A depth measure was obtained using a graduated depth-stick and entered into a field notebook.

For each sampling day, six one-litre bottles (Nalgene I-Chem Certified Pre-Cleaned Wide-Mouth HDPE (high-density polyethylene) Bottles, Cole-Parmer, USA) were pre-labelled to indicate the sample site location and date. Water samples were collected using a dipping method. The bottles were submerged approximately 15 cm below the surface when water depth allowed. In the field, the bottle lid was removed only while the sample bottles were submerged. After collection, the samples were stored in an ice-packed cooler for no more than six hours and transported to the USUHS Water Lab in Bethesda, MD.

Date, time, and local air temperature were recorded at each sampling site. Air temperature was obtained from the "2016 The Weather Network©" cell phone application (Version 4.1.0.923). When appropriate, notable remarks pertaining to each site were recorded in a field lab notebook.

Laboratory Procedures, USUHS Water Laboratory

Retentate Collection for PCR Analysis

Within six hours of being collected, the water samples were processed at the USUHS water lab. Approximately 50 ml from each water sample was filtered through a 0.2 µm pore size, 47 mm filter paper (Polycarbonate Membrane Filters, Nuclepore Track-Etched Polycarbonate (Hydrophilic) Membrane, GE Healthcare) using a porcelain Buchner Funnel (with a fixed perforated plate) vacuum-flask set-up (Figure 9). Each retentate-containing filter paper was placed into a separate freezer bag (2 mil Minigrip Zip-Top Reclosable Bags, Thomas Scientific) and cut, using scissors, into three sections (one half and two quarter pieces). The retentate-containing filter papers were each then placed in a -80^{o}C freezer for storage prior to being transported to the Microbiome Analysis Center (MBAC) at George Mason University (GMU) (Manassas, VA) for DNA extraction.

Between each filtering procedure, the funnel, scissors, and tweezers (used to manipulate the filter paper) were sterilized in a three-step process. The instruments were first sterilized by submerging in a 1:10 solution of 5% sodium hypochlorite bleach (Clorox®) and deionized (DI) water, then rinsed by submerging in tap water, and then rinsed again in fresh DI water (Figure 9). All glassware (funnels and beakers), tweezers, and the Buchner funnel were pre-autoclaved prior to the start of each sampling day.

Figure 9. Buchner Funnel and Sterilization Rinses

E. coli and Total Coliform Analysis

As noted above, the U.S. EPA currently recommends enumeration of E. coli and enterococci as the FIB of choice for fresh recreational water (44; 56). The U.S. EPA's recommended WQS for fresh recreational water is a five-sample, geometric mean of 33 colony-forming units (CFU)/100 ml for enterococci and 126 CFU/100 ml for E. coli *(29; 56)*. A U.S. EPA-approved method of enumerating E. coli and total coliforms in ambient water is the Colilert-18 most probable number (MPN) (CFR 40, Sec 136).

E. coli and total coliform concentrations were determined using Colilert™-18 test kits and the Quanti-Tray™ enumeration procedures (IDEXX Laboratories, Inc.) according to manufacturer's guidelines. Various combinations of non-diluted samples, 1:100, or 1:1000 dilutions were used, depending on the predicted bacterial counts based on the precipitation events in the days preceding the sampling event. All test procedures were conducted after allowing the water samples to reach room temperature.

For non-dilution tests, 100 ml of sample water was collected in the sterile Colilert™ bottle. For 1:100 dilutions, one millilitre of sample water was pipetted into 99 ml of DI water. For 1:1000 dilutions, 0.1 ml of sample water was pipetted into 99.9 ml of DI water. The Colilert™-18 reagent was added to the bottle and the mixture was agitated until all reagent crystals were dissolved. The mixtures were allowed to settle, to reduce foam, and then poured into the Quanti-tray™ package and sealed using the Quanti-Tray™ Sealer. The Quanti-Tray™ packages were then placed in an incubator, set at 35^{o}C, for 18-22 hours. Total coliform counts (number of yellow squares) were recorded. *E. coli* counts (number of fluorescing squares) were counted under UV light and recorded. The number of positive wells was then compared to the manufacturer-provided table to obtain a MPN (23). The MPN provides an estimate of the concentration (CFU/100 ml) of bacterial colonies (either E. coli or Coliforms, respectively) present in the sample mixture.

Turbidity

Each water sample bottle was inverted ten times and then funneled into a vial (specialized for use with the Hach 2100Q Portable Turbidimeter). The external surface of each vial was then wiped clean using a Kimtech KimWipe and then coated with a thin layer of silicon oil, as per Hach 2100Q guidelines. Each vial was then inverted ten times prior to being placed in the turbidimeter. Turbidity readings, in Nephelometric Turbidity Units (NTU), were recorded for each sample. A separate, clean funnel was used for collecting each vial of sample water.

Rain Data

Historical daily rain data for the periods of 16 July to 20 September 2016 and 2-7 February 2017 were obtained from the online archive from the Dickerson Weather Station (12) and "WeatherUnderground" (The Weather Company) (53). The latter was used for analysis as rain data was obtainable from weather stations located in the vicinity of each of this study's six sampling sites (Table 8). The average rainfall across all six sites was calculated and used for analyses.

A significant rain event was defined above as one in which there is at least 0.5" of rainfall within a 24-hour period. There is a corresponding period of elevated discharge into the Creek following a precipitation event. The periods of elevated discharge, calculated as those days for which the daily average discharge is greater than the average monthly flow, were used to define the significant rain event periods. Similarly, low-discharge periods were defined as those days during which the daily average discharge was less than the average monthly flow.

Table 8. Weather Stations

Sampling Site	Weather station name	Station ID:	Location (Latitude, Longitude)
A	Gaithersburg	KGAI	39.17, -77.16
B	Twinbrook	KMDROCKV12	39.08, -77.12
C and D	Jeffry St	KMDSILVE73	39.07, -77.07
E	Rock Creek Palisades	KMDKENSI4	39.04, -77.0
F	Rock Creek Knolls	KMDCHEVY5	39.00, -77.07

Hydrographic Data

Historical daily discharge (flow) rates, for the period of 16 July to 20 September 2016 and 2 February – 7 February 2017, for the Joyce Road Gage Station (ID:01648010) were obtained from the USGS National Water Information System website (60). The

monthly average discharge quantities were calculated and used as the monthly average discharge values. Discharge levels above and below the calculated monthly average were categorized as "elevated-discharge periods" and "low-discharge periods", respectively.

Land Use Data

Based on their geographic location, each of the six sample sites were categorized into one of the three applicable influenced land-use categories: low-density residential, medium-density residential, or recreational land, as determined by Cintron (10) (See Table 6).

Sewer System Data

Due to time constraints, sewer system data was not obtained for analyses.

DNA Extraction and PCR Procedures, MBAC Laboratory

DNA Extraction Protocol

The retentate-containing filter papers, which had been stored at the USUHS Water Laboratory, were transported, on-ice, to the MBAC. As per the recommendations of the MBAC, the following protocol (using FastDNA^{TM} Spin Kit for Soil) was employed for DNA extraction.

One half or one-quarter (if that filter was notably dirty) of retentate-containing filter paper was added to Multimix 2 Tissue Matrix tube. Each tube was labelled throughout all steps. Sodium Phosphate Buffer (978 μl) and MT Buffer (122 μl) was added to each sample tube. Tubes were secured tubes in FastPrep Instrument (bead-beater) and processed for 2x20 seconds at speed 5.5. Tubes were then centrifuged at ~14,000 rpm for 14 minutes. The supernatant was then transferred to a clean tube using a

pipette. PPS reagent (250 μl) was then added and mixed by shaking the tube by hand 10 times. The tubes were then centrifuged at ~14,000 rpm for 5 minutes to pelletize the precipitate. The supernatant was then transferred to a clean 2 ml tube. Well-mixed Binding Matrix reagent (900 μl) was then added to the supernatant. The tubes were then inverted by hand for two minutes to allow binding of DNA to matrix. The tubes were then placed in a rack for three minutes to allow settling of the silica matrix.

Approximately 1300 μl of supernatant was removed (being careful to avoid the settled Binding Matrix) and discarded. The tubes were then agitated using the vortex (for approximately three seconds) to re-suspend the Binding Matrix in the remaining amount of supernatant. Approximately 600 μl of the mixture was transferred to a Spin Filter and catch tube and centrifuged at ~14,000 rpm for one minute. The Catch Tube was then emptied and the remaining supernatant was added to a Spin Filter and re-spun at ~14,000 rpm for one minute. Five hundred microliters of SEWS-M (with 100 ml of 100% ETOH added) was added to the Spin Filter and centrifuged at ~14,000 rpm for one minute. The flow-through was decanted and the Spin Filter replaced in the Catch Tube. The previous step (SEWS-M wash) was repeated and then the matrix of residual SEWS-M wash solution was centrifuged at ~14,000 rpm for two minute to "dry" the matrix of residual SEWS-M wash solution. The Spin Filter was removed and placed in fresh kit-supplied Catch Tube. The Spin Filter was then air dried (with the lid open) for five minutes at room temperature. DES water (150 μl, pre-warmed to 65ºC) was then added to the tubes and vortexed to re-suspend the silica for efficient elution of the DNA. The tubes were then left at room temperature for two minutes. The tubes were then centrifuged at

~14,000 rpm for one minute to transfer eluted DNA to the Catch tube. Eluted DNA was then pipetted to a PCR tube and stored at -20°C for later processing.

Traditional PCR Analysis

To confirm that the extracted DNA was viable and to determine the appropriate dilutions and primers required for qPCR, samples were first analyzed via traditional PCR. The "master mix" for traditional PCR was prepared as follows:

Step:		**Quantity (µl)**
1	DEPC H_20	7.9
2	Add: 10X Rx. Buffer	2
3	Add: 25mM Mg mix	2
4	Vortex mixture	N/A
5	Add: 0.1% BSA	2
6	Vortex mixture	N/A
7	Add: Taq Gold Polymerase (5 units/µl)	0.1
8	Mix mixture by flicking	N/A
9	UV Mix for 4 minutes	N/A
10	Add: dNTPs (2 mM each)	2
11	Add: Forward Primer (10 µM) (see Table 9)	1
12	Add: Reverse Primer (10 µM) (See Table 9)	1
13	Mix mixture by flicking	N/A
14	Aliquot the mix into PCR tubes	N/A
	Total Master Mix	18

Two microliters of each extracted DNA sample was added to 18 µl of master mix, giving a final quantity of 20 µl for PCR analysis. The thermocycler (2720 Thermocycler System, Applied Biosystems by Life Technologies, version 2.09) protocol was set for a volume of 20 µl, annealing temperature of 48°C (with a hot-start at 95°C), for 32 cycles.

Step	**Temperature (°C)**	**Time (minutes)**
1.	95	11
2. 32 cycles	95	0.5
	48	0.5
	72	1
3.	72	10
4.	4	Hold-time

Blue loading dye (5 µl) and 4 µl of extracted DNA were added to each well in preparation for electrophoresis in 1% agarose gel. The power supply (EPS-2000 Series III from CBS Scientific, Inc., Del Mar California) was set to a maximum of 200 V, 150 mA, and 50 W for 30 minutes.

The agarose gel electrophoresis was imaged using a Spectroline view-box (Model TR-312A Transilluminator, 312 nm ultraviolet) and analyzed using Carestream Software (Gel Logic 112, Molecular Imaging Software, Standard Edition, version 5.0.07.22).

An iterative process was used to determine the optimal forward and reverse primers to use for qPCR. Traditional PCR was conducted using various combinations of forward and reverse primers and the results observed via gel electrophoresis (Table 9). Once the presence of DNA was confirmed and the most suitable primers determined, samples were analyzed using qPCR. The gel electrophoresis images can be seen in Appendix C.

Table 9. Traditional PCR Determination of Primers

Traditional PCR	**Figure**	**Lanes**	**Forward Primer***	**Reverse Primer***	**Result**
Run 1	C1	1-6	GenBactF3	GenBact4R	Detected
		17-22	HF183	708r	Undetectable
Run 2	C2	1-21	L27F	355r	Detected
Run 3	C3	1-6 (1)	HF183	H241R	Undetectable
		1-6 (2)	H160F	H241R	Undetectable
		1-6 (3)	H193p	355r	Detected
Run 4	C4	1-21	H193p	355r	Detected

*Primer	Oligonucleotide sequences, 5'-3'	Reference
GenBactF3	GGGGTTCTGAGAGGAAGGT	(47)
GenBact4R	CCGTCATCCTTCACGCTACT	(16)
HF183	ATCATGAGTTCACATGTCCG	(41)
708r	TACCCCGCCTACTATCTAATG	(41)
FAM 27F	AGAGTTTGATCMTGGCTCAG	(49)
355R	GCTGCCTCCCGTAGGAGT	(49)
H241r	CGTTACCCCGCCTACTATCTAATG	(25)
H160f	TGAGTTCACATGTCCGCATGA	(24)
H193p	6-FAM-TCCGGTAGACGATGGGGATGCGTT-TAMRA	(24)

Quantitative PCR

The GenBac3 (GenBactF3, GenBact4R) and BacHum (H193p, 355R) assays were selected for qPCR as the results of the traditional PCR trials indicated they would be most likely to enable amplification/quantification of the respective Bacteroides 16S rRNA genetic markers. The GenBac3 assay, developed by Siefring et al (47), has been reported to be 100% sensitive and specific to the presence of Bacteroides from all warm-blooded animals (31). A recent study by Odagiri et al (31) showed BacHum was the "best" (combination of specificity and sensitive) among five other commonly used assays in detecting human Bacteroides genetic markers (Table 10).

Table 10. Real-Time PCR Assays

Assay name	Reported organism (target)	Primers
GenBac3	Total Bacteroides Species (1)	GenBactF3
		GenBact4R
BacHum	Human-associated Bacteroides (2)	H193p
		355R

Reference: Odagiri et al (31), Supplemental Material

The qPCR was run using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, ThermoFisher Scientific**,** Catalog number: 4376600) in series with StepOne™ Software (Version 2.2.2, Applied Biosystems, Life Technologies). The

"master mix" used for qPCR was a pre-prepared formula: PowerUp™ SYBR® Green Master Mix (Applied Biosystems™, ThermoFisher Scientific, Catalog number: A25742). The specific qPCR protocol and experimental details are included in Appendix D.

PCR Quality Control Measures

MBAC's standard PCR lab procedures were employed to avoid sample contamination and the qPCR assays were conducted in accordance with the internal validation methods used by the MBAC. DNA from Bacteroides fragilis (product number 25285D, ATCC, Manassas, VA); Bacteroides thetaiotaomicron (product number 29148D-5, ATCC, Manassas, VA); E. coli, which was cultured at MBAC from a Cloning Kit (TOPO TA Cloning®, ATCC #9637, S. A. Waksman), and human stool samples (from a storage supply at MBAC) were used as positive controls. Negative controls (i.e. "master mix" with no DNA added) were analyzed to rule out inadvertent DNA contamination.

Additional Methods

Due to methodological problems in the collection of PCR data, which went undetected until very late in the process of this study, modifications were required to salvage this study. As will be discussed in greater detail in the Results and Discussion Section, despite various attempts, DNA was not detectable by traditional PCR in any of the original 126 samples (collected from 20 July – 20 September 2016). Experiments were run using undiluted and 1:5 dilutions from both the residual from the binding matrix and then from purified DNA. The process was then repeated using the unused portions of the frozen filter papers. The DNA extraction process was repeated and traditional PCR analysis run. Because this failed to yield detectable DNA, yet the positive controls were

detected, it was concluded that the sample filters papers did not contain bacteria and/or DNA in sufficient quantities to be detected via PCR. The possible reasons for this will be discussed below in the Results and Discussion Section.

On 7 February 2017, in an attempt to obtain detectable DNA samples, 2 L of sample water were collected from each of the six sampling sites. The previously described procedures and data collection methods, with the exceptions as noted below, were repeated for this additional sampling day. In contrast to the filtering procedures used previously, the water samples were transported, on ice, directly to the MBAC Laboratory. The water samples were refrigerated overnight and then filtered, using the Buchner funnel filter-flask apparatus shown in Figure 10, using the water quantities listed in Table 11 below, according to the observed "dirtiness" of the filter paper. Traditional PCR was conducted, as described previously, followed by qPCR.

Figure 10. Buchner Funnel Filter Flask Apparatus

Table 11. Quantities of Sample Water Filtered

Site	Amount of sample water filtered (ml)
A	250
B	200
C	300
D	200
E	200
F	200

Statistical Analysis

All statistical analyses was conducted using IBM® SPSS® Statistics versions 22 and/or 24 and Microsoft Excel 2013, in consultation with the USUHS Biostatics Consulting Center. All statistical tests were performed at a significance level (p) of $p<0.05$ (two-sided). A Shapiro-Wilk test was used to determine whether each of set of water quality data was normally distributed. If the data set was determined to be non-normal, non-parametric statistical tests were used. Descriptive statistics (mean, range, and confidence interval) were calculated to characterize, and compare to the regulatory standards, the data collected for standard water quality parameters (DO, pH, temperature, TDS, conductivity, and turbidity). The following descriptive and inferential statistical methods were applied to each of this study's stated research objectives:

Primary research question: Is the proportion of human fecal contamination in RC less than or equal to the last known measure of 10.5%?

Statistical method: The average proportion of human Bacteroides markers ([BacHum]/[GenBac3]) across all sampling sites was calculated, where [BacHum] is the concentration of human-associated Bacteroides DNA per sample (ng/μL) and [GenBac3] is the concentration of all warm-blooded animal-associated Bacteroides DNA per sample

(ng/μL). It is important to note that GenBac3 concentrations will include human-associated DNA and represents the total number of detected Bacteroides species.

A sample size of 120 (6 sites x 20 sampling days) was calculated to be sufficient to estimate an overall concentration and percent human fecal concentration with a margin of error (MOE) or 0.18 standard deviations (SD) overall, an MOE of 0.47 SDs for each site (N=20), and an MOE of 1 SD for each day (N=6) based on a 95% two-sided confidence interval for a mean (30).

Secondary research questions:

1. Is there a correlation between the quantity of E. coli detected via traditional methods and the proportion human Bacteroides genetic markers detected via qPCR in RC?

   Statistical Method: If data sets displayed a normal distribution, the Pearson test was employed and if a data set was determined to be non-parametric, the Spearman's test was used to determine whether a correlation exists.

2. Do significant rain events lead to an increased quantity of E. coli and/or proportion of human-associated fecal contamination in RC?

   Statistical Method: Discharge rates were used as a surrogate for rain events. E. coli concentration data was not normally distributed. After log-transforming the E. coli concentration data, the concentration during low-dicharge event periods remained non-normal; therefore non-parametric statistics were applied. An unpaired Mann-Whitney U test was used to determine whether there was a statistically significant difference between the distribution of the geometric mean

E. coli concentration during elevated-discharge periods and low-discharge periods.

3. Is there a relationship between influenced land-use type and E. coli and/or the proportion of human-associated fecal contamination levels in RC?

   Statistical Method: Again, because the E. coli concentrations were not normally distributed, a nonparametric test was applied. The daily geometric mean E. coli concentrations for all sites within a particular land-use category were calculated. The Friedman (paired, nonparametric) statistical test was used to determine whether there existed a significant difference across the three different influenced land-use categories. The data were considered "paired" as all three land-use categories were compared on the same dates.

4. Is there a correlation between older (unrepaired) sections of the main sanitary sewer lines adjacent to RC and the proportion of human-associated fecal content in RC?

   Statistical Method: Sewer data was not collected due to time constraints.

CHAPTER 3: RESULTS AND DISCUSSION

Water Quality Standards

The mean values for standard water quality parameters were calculated across all sites, along with their respective confidence intervals, and compared to the regulatory standards (Table 12). The standards reflect the Code of Maryland Regulations (COMAR) or recommended values from published studies (10). For temperature calculations, further granularity was required. The average temperature for sites falling within each designated use class category was calculated and compared to the standard for that particular Class. Sites B-F fall within Class I waters, whereas Site A is within Class 3 waters.

Table 12. Comparison with Water Quality Standards

Parameter	**Standard**	**Mean**	**Max**	**Confidence Interval**
Conductivity (µS)	< 600	339.6**	485.3	300.0 - 379.1
Dissolved Oxygen (mg/L)	> 5	6.8	5.9 (min)	6.5 - 6.9
E. coli (CFU/100 ml)	**126**	**1598.5***	**16547***	**-18.2 - 3215.2**
pH	6.5 - 8.5	7.4	7.7	7.3 - 7.5
Temperature (ºC) :				
- Class 1 waters	< 32	23.8	25.9	23.1 - 24.6
- **Class 3 waters**	**< 20**	**20.9***	**22.9***	**20.3 - 21.6**
Total Coliforms (CFU/100 ml)	N/A	59695.0	546737	7293.7 - 112096.2
Total Dissolved Solids (mg/L)	< 1000	178.1**	242.5	157.6 - 198.5
Turbidity (NTU)	< 150	12.0	94.7	3.0 - 21.0

*Standard exceeded; **Normally distributed data; n = 21

As can be seen in Table 12, all of the parameters, with the exception of E. coli concentration and water temperature at Site A, are within the regulatory standards. The finding of elevated E. coli concentrations supports the current designation of Rock Creek as impaired due to elevated levels of fecal bacteria. In discrepancy with a 2012 report by Biohabitats Inc (3), conductivity was determined to be within the regulatory standards. Graphical results for each of the standard water quality parameters are presented in Appendix E.

To determine whether a correlation exists between rainfall quantity and discharge rate, a cross-correlation procedure was used. The strongest correlation ($r = 0.832$, $n = 67$) was found to occur with a one-day lag period between rainfall event and elevated discharge (i.e. high discharge events occur one day later than high rainfall events). Figure 11, showing discharge (ft^3/sec) and daily rainfall plotted against time, illustrates the lag time between discharge and rainfall. The strong correlation confirms the assumption that the rain gauge stations selected to assess precipitation levels truly demonstrate that the rainfall events were effecting Rock Creek. The observed lag time is likely due to a combination of factors. Because the rain gauge stations were not located at the sampling sites, rainfall that was recorded at the stations may not have fallen at the sampling site and could have fallen anywhere within the watershed upstream of the flow gauge station, therefore a lag would exist as there would be travel time required for the rainfall to accumulate and travel (e.g. via run-off, storm sewers, or through ground) into the creek. Additionally, because the discharge rate gauge station was located approximately five kilometers downstream from the southernmost sampling site (Site F), this would also cause a lag between rainfall and discharge data. Owing to the distance between sampling sites and the gauge station, the discharge quantity would be elevated, relative to what it would be if it were located at the sampling sites, as more water is added to the creek as it flows south.

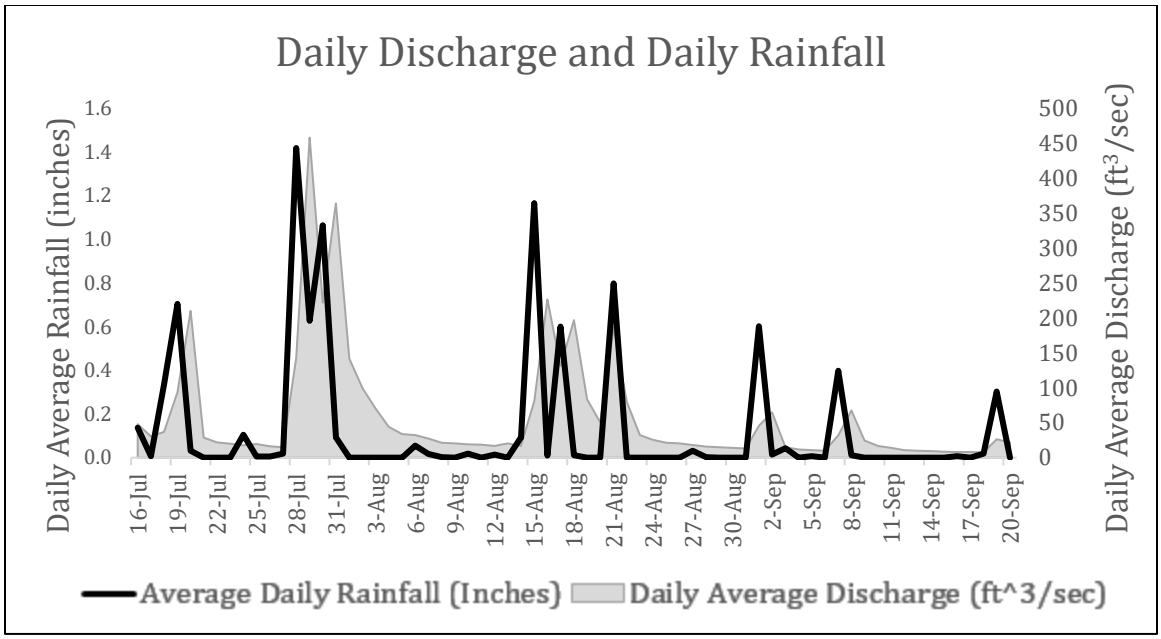

Figure 11. Daily Discharge and Daily Rainfall

Microbial Water Quality and Anthropogenic Factors

The concentrations of GenBac3, BacHum, and E. coli and the calculated proportion of human Bacteroides genetic markers for each sampling site for the samples collected on 7 February 2017 are reported in Table 13 and shown graphically in Figure 12. It is important to note that, unlike the findings throughout the original sampling period, each of the E. coli levels from the February sampling day are within the COMAR standard of 126 CFU/100 ml. This finding is not unexpected, as E. coli levels typically decrease during the colder seasons (4); however, the effect the cold weather and seasonality would have on the *proportion* of human Bacteroides genetic markers in this study area is unknown.

While Bacteroides appear to be less sensitive to cold temperatures than E. coli (20), in the current study, E. coli is being measured via culture methods (which necessitate live bacteria), whereas the Bacteroides concentration is determined via PCR

(which detects DNA from both and live and dead cells) (8). The implication of this is that, in addition to the differing survivability rates, E. coli measurements (by culture methods) will be affected by cold weather whereas Bacteroides measured by qPCR will not.

Additionally, it is noted that for Site A the percent human-associated Bacteroides marker is > 100%, which is a nonsensical result suggesting that there is a greater quantity of human-associated fecal contamination than the total fecal contamination (which includes human fecal contamination). The most plausible explanations for this result are that it is either due to laboratory error or that the BacHum marker is sensitive to some non-human sources of Bacteroides. While BacHum has been demonstrated to be 100% sensitive to sewage detection, the same study showed that BacHum has cross-reactivity with some animals, notably, chicken (70%), buffalo (10%), and goat (10%) (31). Additionally, an earlier study showed cross-reactivity of BacHum with dog feces (12.5%) (24). Because Site A is located in the northern, more rural region of Montgomery County, where there is more agriculture, it is possible that the BacHum assay did detect chicken, goat, or dog feces, which would artificially increase the concentration of BacHum markers. While the possibility of observing artificially elevated "human-associated" Bacteroides levels is possible, the GenBac3, which is reported to be 100% sensitive to all warm-blooded animals (31), should also detect the non-human Bacteroides, thereby maintaining a proportion ([BacHum]/[GenBac3]) of less than 100%. Therefore, unless the GenBac3 assay is less sensitive than reported, cross-reactivity alone does not fully explain the resulting ratio being greater than 100%.

Table 13. Bacteroides DNA and E. coli Concentrations

Site	[BacHum] Average (ng/µL)	[GenBac3] Average (ng/µL)	Percent Human Markers ([BacHum]/ [GenBac3]) (%)	E. coli Level (CFU/100 ml)
A	1.96E-03	1.28E-03	153.2	24.6
B	0.833E-03	1.43E-03	58.4	20.1
C	1.52E-03	2.14E-03	70.9	77.1
D	0.50E-03	0.75E-03	66.4	95.9
E	0.79E-03	1.47E-03	53.5	70.8
F	0.86E-03	2.38E-03	36.2	53

* All results from 7 February 2017 sampling day only

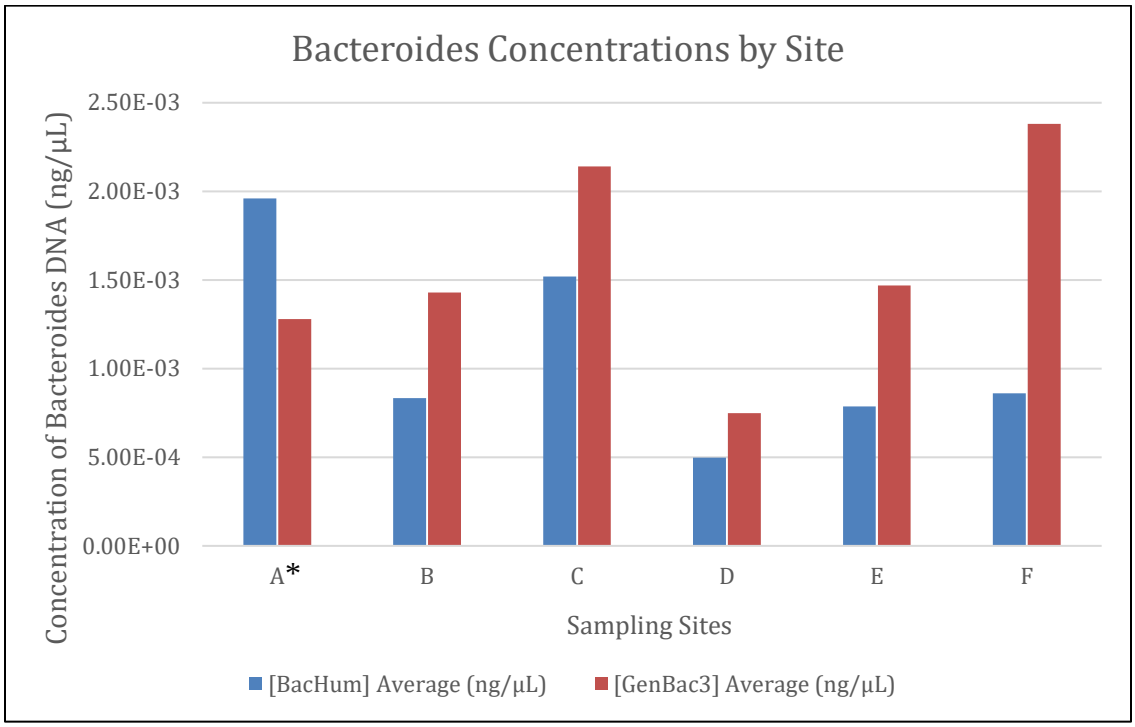

Figure 12. Bacteroides Concentrations by Site
*Outlier Site A; All results from 7 February 2017 sampling day only

The mean proportion of human-associated Bacteroides markers was calculated to be 73% (CI: 30-120%). When excluding the outlier (Site A) data, the proportion is reduced to 57% (CI: 40-74%). Note that the lower limit of the proportion of human Bacteroides markers ([BacHum]/[GenBac3]) in both cases is much greater than the

previous reference value of 10.5% human FIB (Table 14). The mean human and all-warm-blooded animal Bacteroides concentrations are shown in Figure 13.

Table 14. Bacteroides Concentrations

	N	Mean	Confidence Interval	
[GenBac3] (ng/µL)	6	1.6E-03	9.5E-04	2.2E-03
[BacHum] (ng/µL)	6	1.1E-03	5.0E-04	1.6E-03
[E. coli] (CFU/100 ml)	6	57	25	89
Ratio ([GenBac3]/ [BacHum])*	6	73%	30%	120%
Excluding Site A:				
[GenBac3] (ng/µL)	5	1.6E-03	8.3E-04	2.4E-03
[BacHum] (ng/µL)	5	9.0E-04	4.3E-04	1.4E-03
[E. coli] (CFU/100 ml)	5	63	28	99
Ratio ([BacHum]/[GenBac3])	5	57%	40%	74%

*Non-normal distribution

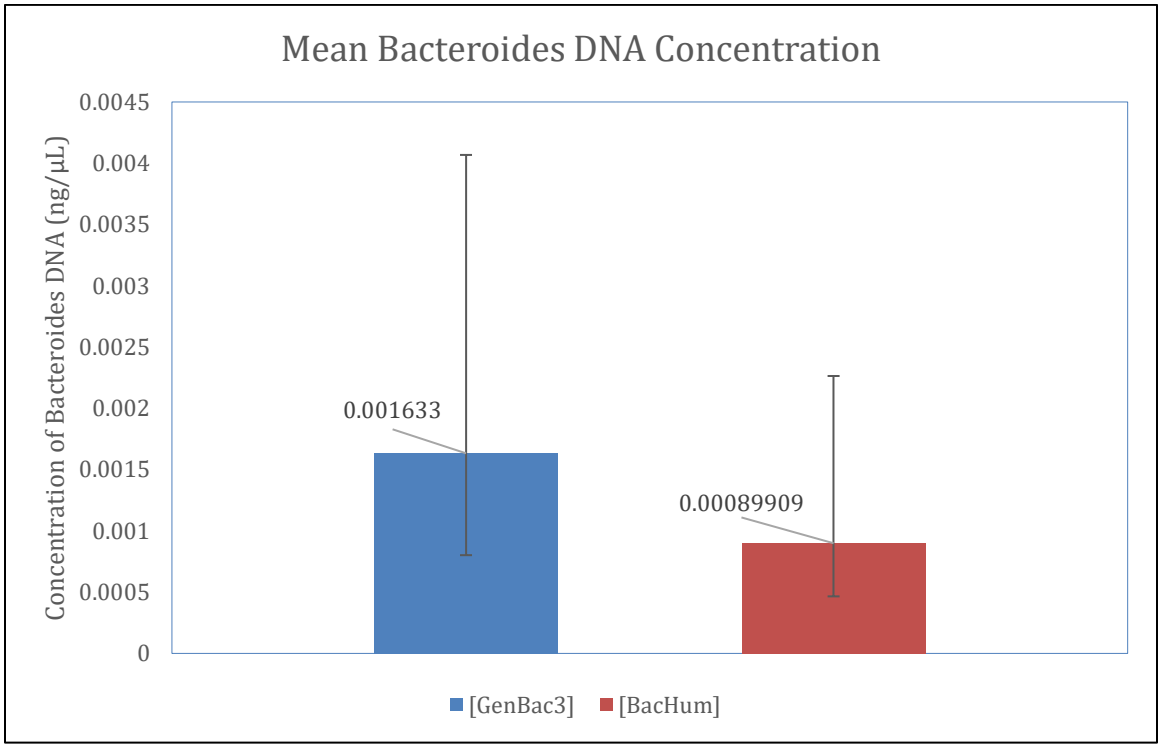

Figure 13. Human and All-Animal Bacteroides Concentration

As shown in Table 15, the Pearson test showed very poor, non-significant correlation between GenBac3, BacHum, and E. coli concentrations at each site (Figure

14). The data for GenBac3, BacHum, and E. coli levels in February were all normally distributed; therefore parametric tests were used for analyses.

Table 15. Correlation Table: GenBac3, BacHum, and E. coli Concentrations

	Correlation (r)	Significance Level (p)
GenBac3 to BacHum	0.220	.675
GenBac3 to E. coli	-0.115	.828
E. coli to BacHum	-0.442	.381

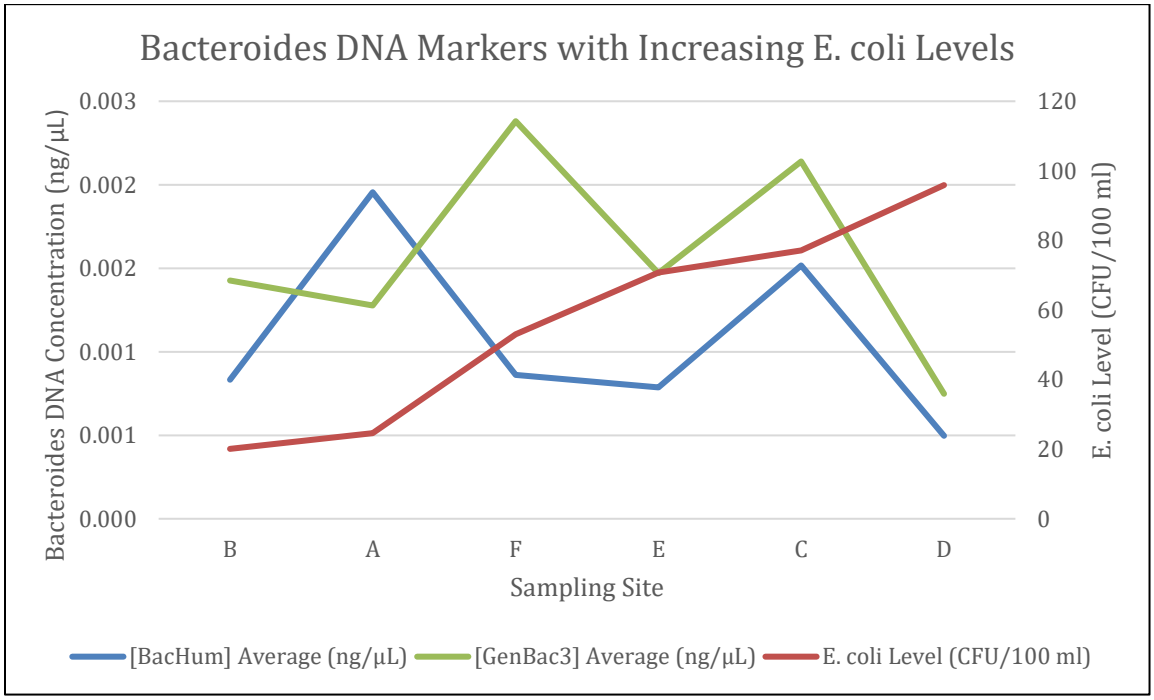

Figure 14. Bacteroides Markers with Increasing E. coli Levels

The relationship between E. coli and human Bacteroides proportion was investigated using Spearman's rho correlation coefficient. Percent human Bacteroides marker is not distributed normally; therefore a nonparametric statistical test was conducted. There was a very small, non-significant, positive correlation between the two variables, r = 0.086, n = 6, p = 0.872. A correlation test was conducted again with the exclusion of Site A. Because the data is normally distributed with the exclusion of Site

A, the relationship was investigated using the (parametric) Pearson product moment correlation coefficient. There was a moderate, non-significant, positive correlation between the two variables, r = 0.404, n = 5, p = 0.501. In both cases, a greater sample size would be required to obtain greater statistical power. The correlation is presented graphically in Figure 15.

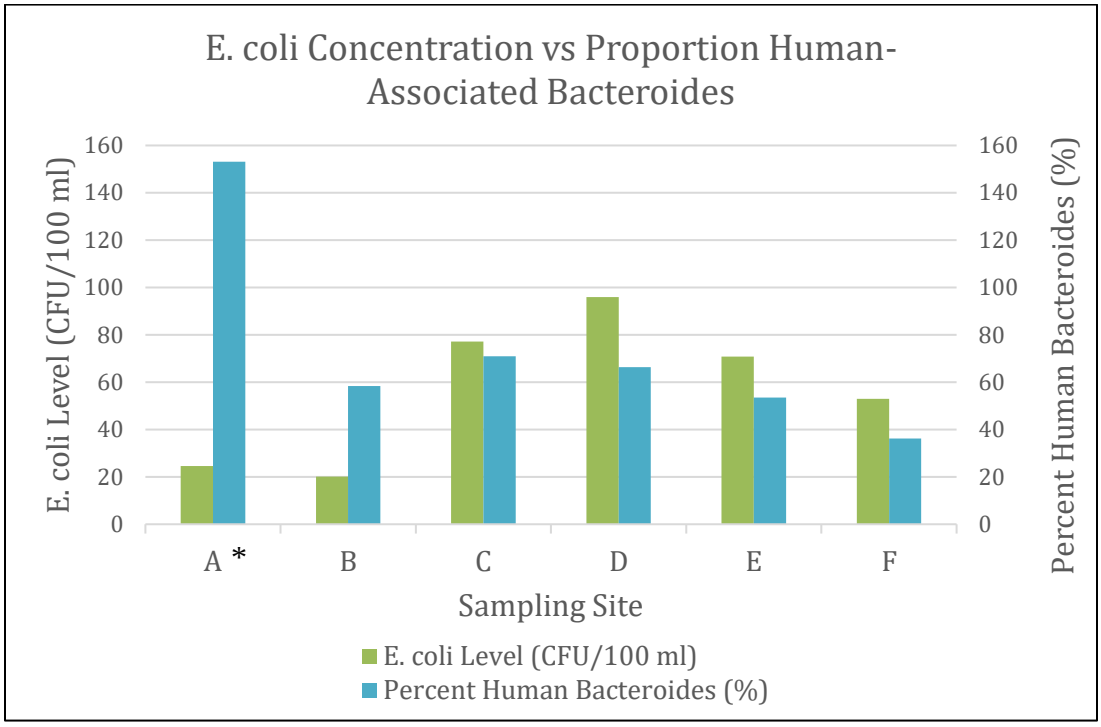

Figure 15. E. coli Concentration vs Proportion Human Bacteroides
*Outlier Site A

While there was insufficient data to determine whether rainfall affects human fecal proportion (no precipitation recorded in the study area for 2-7 Feb 2017 (53)), a Pearson cross-correlation test showed there is a statistically significant, moderate correlation (r=0.545, p=.011, n=21) between rainfall and E. coli concentrations, as illustrated in Figure 16. A spike in E. coli concentrations can be seen following each

instance of significant rainfall (excluding 21 August 2016), and even on days with elevated rainfall not reaching greater than 0.5 inches. Another trend that is evident in Figure 16, but perhaps better visualized in Figure 17, is that with consecutive significant rainfall days, it appears there is a decrease in the rise of E. coli concentration. It is speculated that this trend might reflect that the source of the bacterial contamination is surface run-off, which may become less contaminated as more rain washes away fecal matter found on the ground, as opposed to coming from CSOs or sewer exfiltrations, which would likely provide a continuous source of fecal contamination given significant rainfall. Alternatively, if the spike in E. coli is due to disruption of E. coli that had been sorbed to sediment within the creek, the addition of more rainfall could have the effect of diluting the E. coli levels with subsequent rainfalls.

If it is the case that the elevation in SFIB is from run-off, as suggested by these results, an appropriate follow-up question would ask why there is such a high proportion of human-associated fecal bacteria, which typically would not be associated with run-off. The SFIB data is more reliable as it was measured using a standardized technique, with a sufficient sample size and good spatial and adequate temporal representation. So the apparent conflict between the speculated source of SFIB and the high level of human-associated fecal contamination is likely due to the unreliability of the PCR data being determined one only one day, using a non-standardized technique.

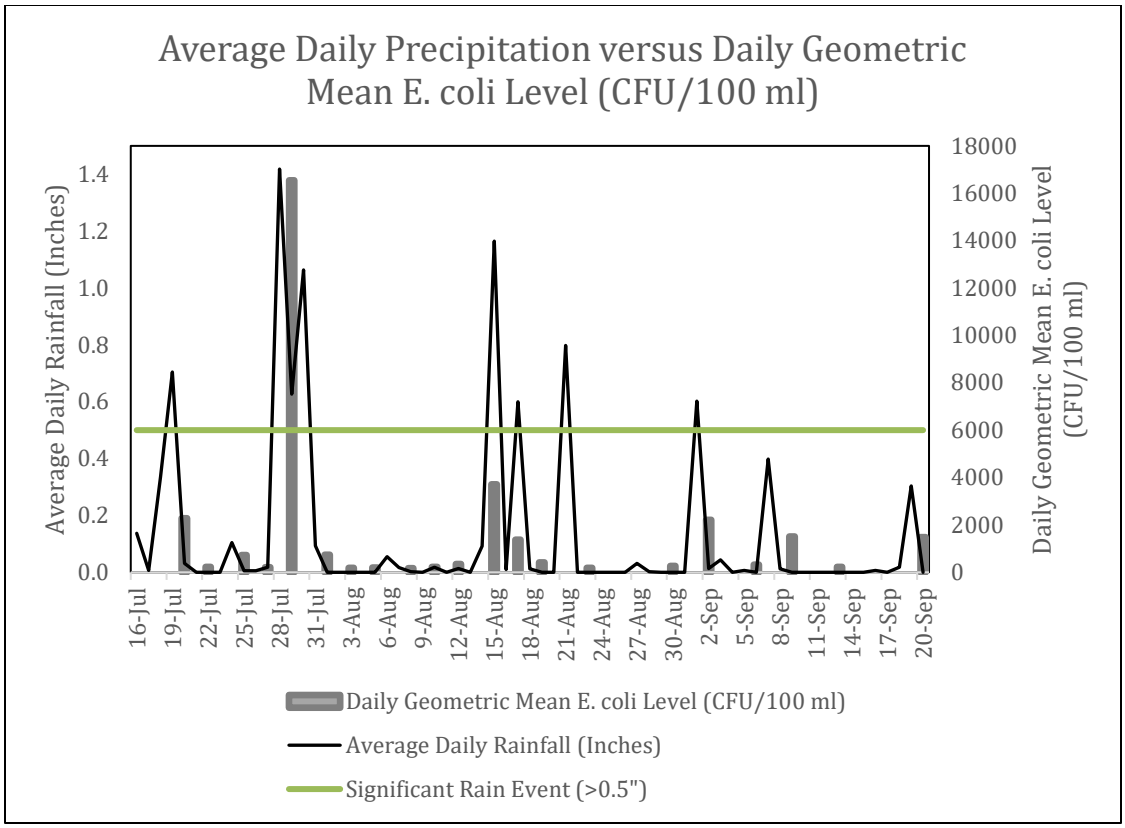

Figure 16. Average Daily Precipitation and Daily Geometric Mean E. coli Level

The results of an unpaired Mann-Whitney test showed there is a significant difference ($p = 0.017$) between E. coli concentrations during elevated-discharge events and low-discharge events. This difference can be visualized graphically in Figure 17. The Department of the Environment (Maryland) reported no sewer overflows in the Rock Creek watershed during the study period; however, one SSO was discovered on 28 June 2016 and discharged an estimated 400 gallons of sewage into Stoney Creek (52). This tributary drains into Rock Creek upstream of sampling Site F only.

There is an E. coli concentration spike noted on 20 September 2016 which, unlike all other E. coli spikes, does not occur during an "elevated discharge period". The period of 8 – 19 September 2016 is a notably "dry" period. During this period it is expected

that, due to absence of precipitation, the land would become quite dry. The rain that does fall on 19 September 2016, while not significant enough to cause "elevated discharge" because the majority of the rainfall was likely absorbed into the ground, was sufficient to cause a spike in E. coli.

As defined above, an "elevated discharge period", is one in which the discharge rate is greater than the calculated monthly average discharge rate. This reference value (monthly average discharge) was used for ease of calculation; however, it should be noted that the average monthly discharge rate will be greater than the "baseflow". Baseflow is defined as "a portion of streamflow that is not directly generated from the excess rainfall during a storm event. In other words, this is the flow that would exist in the stream without the contribution of direct runoff from the rainfall" (38). So, where monthly average discharge calculations will include the effect of rainfall, baseflow calculations aim to exclude this effect, thus calculated average monthly discharge rates will be greater than the calculated baseflow. The determination of baseflow, however, is not an exact science, and there are many different method which have been used to determine baseflow, based on what have been described as arbitrary criteria (17). The use of baseflow instead of monthly average discharge would likely explain the E. coli spike on 20 September, as it can be observed that the small rise in discharge would likely be above baseflow. The use of average monthly discharge can be seen as a more conservative measure, in that statistical significance was obtained despite the potential of missing rises in discharge as a result of defining elevated discharge by a greater threshold.

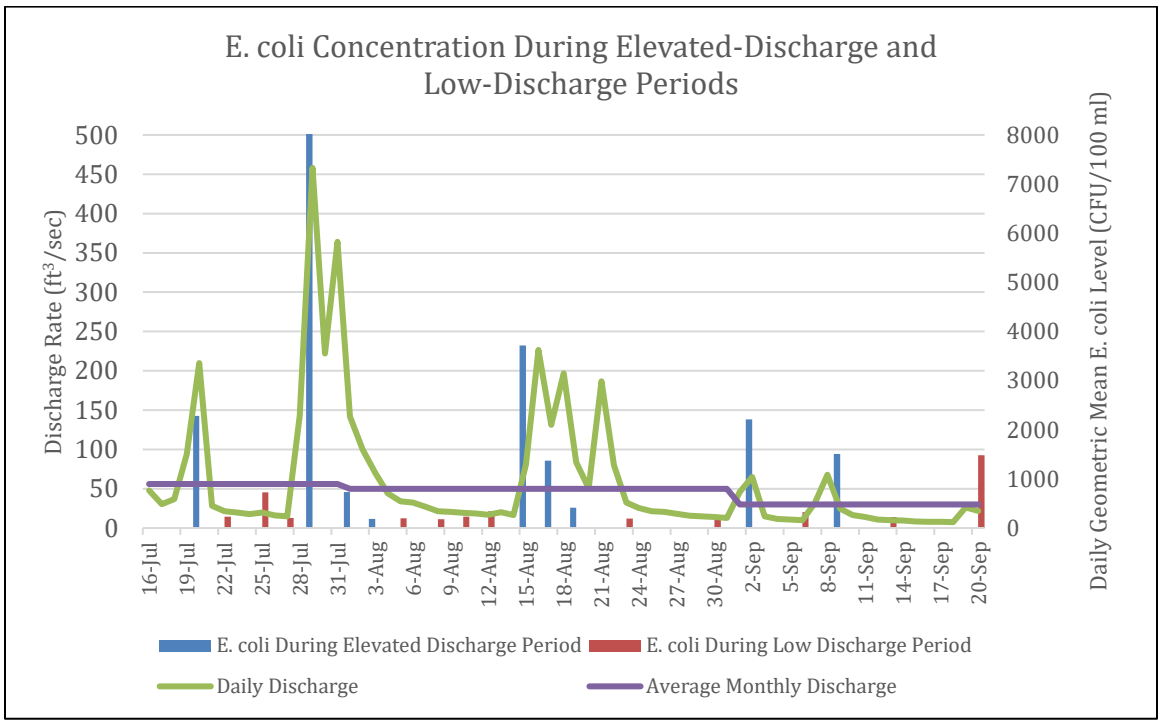

Figure 17. E. coli Concentration During Elevated and Low-Discharge Periods

Results related to the relationship between influenced land-use and the proportion of human-associated fecal contamination are presented using descriptive statistics only as there was an insufficient sample size to conduct inferential statistics. The outlier, and single data point representing the proportion of human-associated fecal contamination level at Site A, was excluded from Figure 18. For E. coli data, the results of the Friedman test indicate there is no significant difference (n=21, p=0.084) in E. coli concentrations across the three influenced land-use types, even with removal of the major outlier (data point representing 29 July 2017; n=21, p=0.142) (Figure 19).

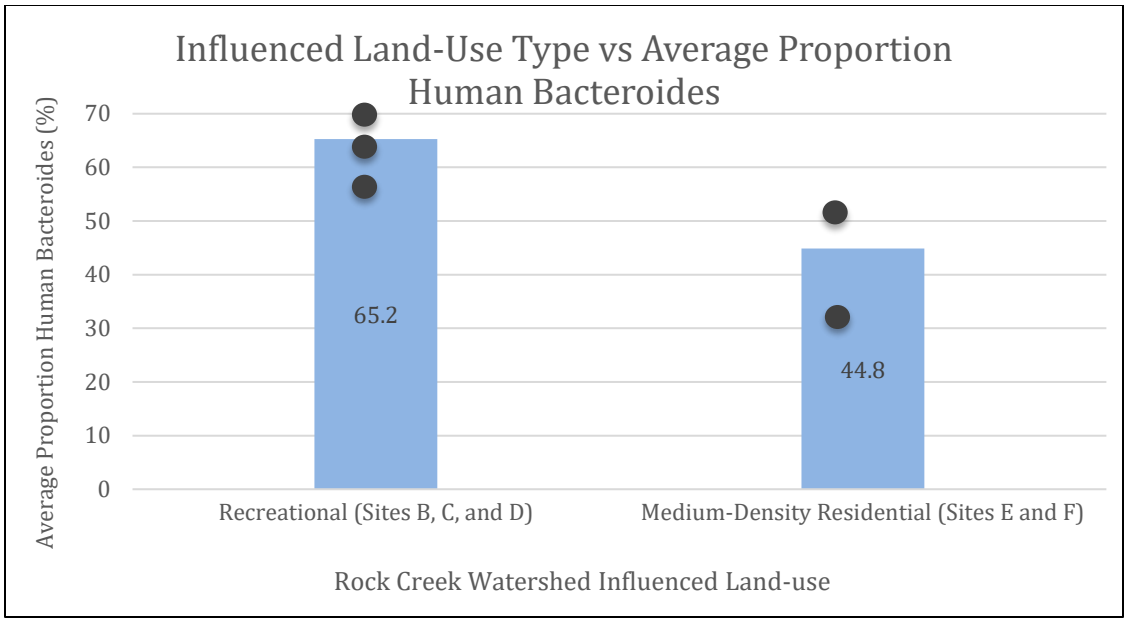

Figure 18. Influenced Land-Use Type vs Proportion Human Bacteroides Markers ($n_1 = 3$, $n_2=2$)

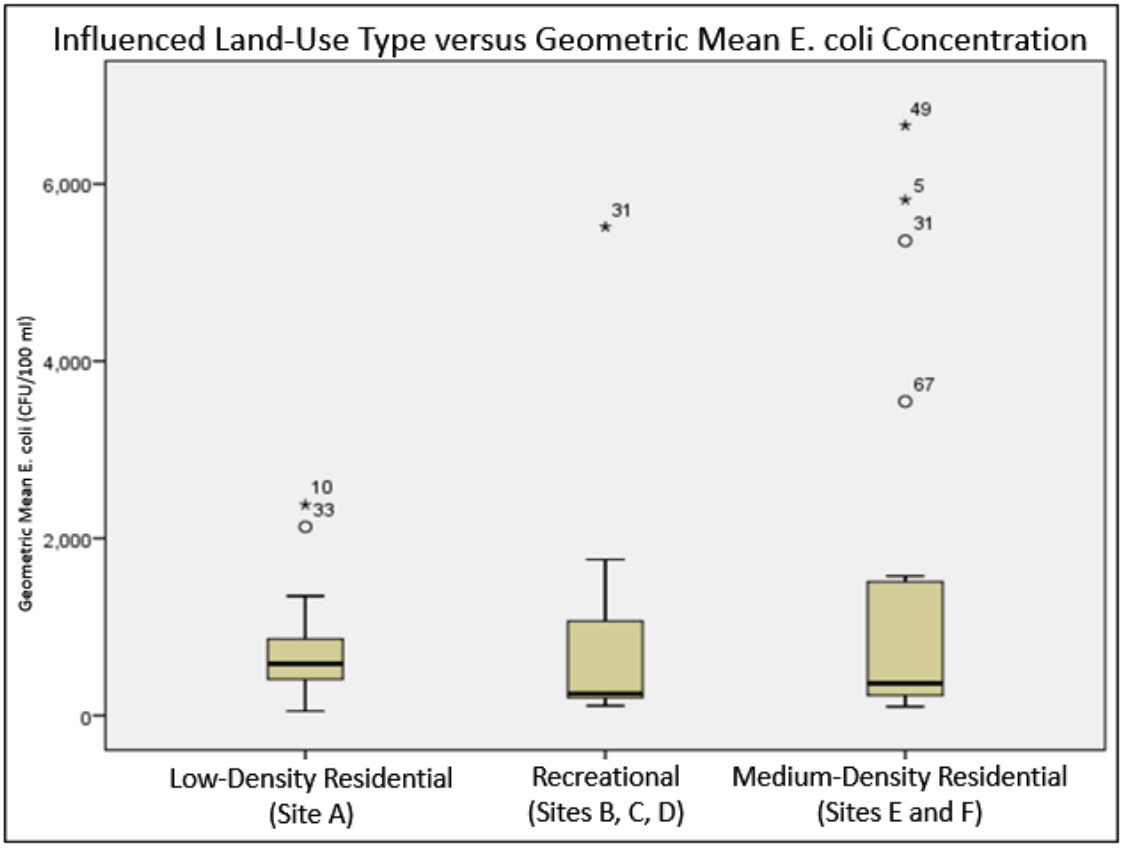

Figure 19. Influenced Land-Use Type vs Geometric Mean E. coli Concentration

CHAPTER 4: CONCLUSIONS

PRIMARY RESEARCH OBJECTIVE:

The primary objective of this study was to determine whether the proportion of human-associated fecal contamination in RC less than or equal to the last known measure of 10.5%. The results indicate that the proportion of human-associated fecal contamination is much greater (by a factor ranging from four to seven) than the previously reported value of 10.5%. This result should be interpreted with extreme caution owing to the multitude of study limitations reported below.

SECONDARY RESEARCH OBJECTIVES:

1. *To determine whether there is a correlation between the quantity of E. coli detected via traditional methods and the proportion of human Bacteroides genetic markers detected via qPCR in RC.* A relationship appears to exist between the proportion of human-associated fecal indicators present, as measured by qPCR, and the concentrations of E. coli as determined by a traditional culture method. The very cautious interpretation, owing to the limitations noted below, of this trend showing that E. coli and the proportion of human-associated fecal indicators have a positive, moderate correlation, is that E. coli monitoring, in this study area, may be a suitable surrogate for gauging human fecal contamination levels.

2. *To determine whether significant rain events lead to an increased proportion of E. coli and/or human-associated fecal contamination in RC.* E. coli concentrations were shown to be significantly different following rain events compared to during dry-weather periods. Sewer overflows did not appear to be responsible for the spikes in FIB levels, thus run-off appears to be a significant contributing factor. Mitigation strategies

designed to reduce the quantity or contamination levels of stormwater run-off may therefore be most effective in reducing FIB levels.

There was insufficient data (i.e. a single sample day with no preceding rain event) to make any determination regarding a relationship between rainfall and human fecal proportions.

3. *To determine whether a relationship exists between influenced land-use type and E. coli and/or human-associated fecal contamination levels in RC.* There appears to be no significant difference in E. coli concentrations across influenced land-use types. A similar result was reported by Cintron (10), who found that there was no correlation between land-use type and enteric bacteria concentration along the length of Rock Creek.

Regarding human-associated Bacteroides, there appears to be a trend indicating the creek is more contaminated with human feces in areas influenced by recreational versus medium-density residential land-use. However, again owing to the small sample size and temporal limitations, a trend is difficult to establish.

4. *To determine whether older (unrepaired) sections of the main sanitary sewer lines adjacent to RC correlate with an increased proportion of human-associated fecal content in RC.* Due to time constraints, sewer system data was not obtained for analyses; therefore no conclusions can be drawn regarding the potential relationship between sewer systems and the proportion of human-associated fecal content in RC.

LIMITATIONS

General Limitations

Sampling is ideally conducted over time and space to best capture a representative profile of the quality of a body of water. While this study was able to collect samples

from a range of locations along the creek, the sampling period spanned only ten weeks (excluding the one additional February sampling day), and further, the samples containing viable (PCR) were collected on only one day. Therefore, great caution must be exercised in drawing conclusions from these findings as water quality assessments based on a one-day sampling event can be dramatically influenced by a multitude of factors (e.g. weather events, temperature, CSOs, sewer exfiltration events, run-off events (sludge release), spills, seasonal variations, animal movement patterns, etc.). In addition, as noted by Tetra Tech Inc. and Herrera Environmental Consultants (51), "because the bacteria isolates analyzed from collected water samples represent a small portion of the population present in the sample (and an even smaller portion of the waterbody population), the results might not represent the actual relative presence of sources in the watershed".

As noted, data regarding the Rock Creek sewer system upgrade was not captured for this study. Therefore the influence that this variable may have had on the water quality of Rock Creek was not accounted for in this study.

PCR-Specific Limitations

Only six of the 132 water samples collected produced sufficiently detectable DNA when amplified via traditional PCR. The potential reasons for being unable to detect DNA in the majority of samples includes: sterilization methods, storage/transport, DNA extraction process error, insufficient quantity of sample water filtered, and sample water bypassing the filter paper. Each of these possibilities will be discussed in detail below.

The sterilization method used at the USUHS water lab included a three-step process between each filtering procedure to sterilize the funnel, scissors, and tweezers (used to manipulate the filter paper). The instruments were first sterilized by submergence in a 1:10 solution of 5% sodium hypochlorite bleach and DI water and then rinsed by submerging in tap water and finally in fresh DI water. The primary concern initially with this step was ensuring that all DNA was destroyed between subsequent filtrations. Because the focus was on preventing DNA cross-contamination, consideration of sodium hypochlorite contamination was neglected. There was no forethought to measure the final fresh DI rinse-water for sodium hypochlorite, which if present, may have destroyed DNA in subsequent filtrations. While this is a possibility, it is unlikely because the first filtration conducted each day would have been processed on equipment that had been sterilized in an autoclave and not via bleach, therefore the first sample of each day would likely have contained detectable DNA.

As described in the Methods Section above, once water samples were collected, they were stored in a cooler, on ice, for no more than six hours, prior to being filtered. The filter papers were then immediately stored in a -80^{o}C freezer. The frozen filter papers were then transported, on ice to the MBAC, for no more than two hours, before undergoing the DNA extraction process. Once extracted, the purified DNA was stored in vials at -20^{o}C for 3-6 months. All of these storage/transport procedures are in keeping with standard methods for preserving DNA samples found in the literature, making this an unlikely explanation for the failure to detect DNA. It was reported that approximately half of the purified DNA samples were inadvertently removed from the -20^{o}C freezer from 10 minutes to 3 days at some point throughout the fall. While this is noteworthy

and could explain some erratic data, it does not explain the failure to detect DNA in all samples, even those that were not removed from the freezer inadvertently.

The multistep DNA extraction process described in detail in the Methods Section is another potential cause for failing to detect DNA. The process includes numerous steps, requiring a number of chemical additives, and very precise quantities. In short, it is a process that has many areas which are vulnerable to error. However, it is felt that these vulnerabilities are negligible for the following reasons: the FastDNA^{TM} Spin Kits that were used are an industry standard, had been used successfully in other experiments, and more than one kit was used over all the samples, so the unlikely chance that a kit contained faulty chemicals, is reduced even further. Also, the lab protocol that was followed is the standard MBAC DNA extraction procedures that has been used extensively and successfully in previous studies. So, while it can't be ruled out that the DNA extraction process is the culprit responsible for the non-detected DNA, it is unlikely.

Based on the recommendation from the staff at the MBAC, 50 ml of sample water was filtered for each sample. In hindsight, it was felt that this quantity of water may have been insufficient to obtain the threshold concentration of bacteria to enable DNA detection via PCR. For reference, Seurinck et al (41) reported the Bacteroides marker limit of quantification, via qPCR using the HF183 primer, was $4.7 \pm 0.3 \times 10^5$ human-specific Bacteroides markers per litre of freshwater, corresponding to a dilution of 10^{-5}. The traditional PCR LOQ was $1.3 \pm 0.4 \times 10^7$ human-specific Bacteroides markers per litre of freshwater, corresponding to a dilution of 10^{-2}. The limit of detection was not determined because DNA still detectable at lowest dilution trialed (10^{-9}).

This theory was tested to some extent as for the additional sampling day (7 February 2017) at least 200 ml of sample water was filtered for each sampling site, resulting in detected DNA. However, this does not make it conclusive that sample water quantity was the issue, because other procedural methods were changed as well for the additional sampling day. Also, there were much higher E. coli and total coliform levels recorded during the warmer months than compared with the February sampling day (by a factor of >10, in some cases). So, despite filtering only one-quarter the quantity of sample water in the July-September water samples compared to the February samples, it is highly unlikely that the bacterial population in the July-September sample water was below the threshold of detectable DNA by traditional PCR if the February samples exceeded the threshold.

On the additional sampling day, the water was transported, on ice, directly to MBAC, where it was stored in a -20^{o}C freezer overnight and then filtered the next day. This was a significant departure from the storage/transport method used previously in the study. Also, and perhaps more significantly, different sterilization and filtration methods than were followed at the USUHS Water Lab were used at the MBAC. At the MBAC, the two glass pieces (not the metal clamp) of the filtration apparatus were submerged in 10% bleach for approximately 1 minute, then submerged in DI water, and dried between each successive filtration. The MBAC laboratory used the two-piece clamped Buchner Funnel, which necessitates that all sample water pass directly through the filter paper because the filter paper is sealed between the two pieces making up the funnel apparatus (Figure 10). In contrast, the Buchner funnel used at the USUHS Water Lab is such that the filter paper rests on the surface of the fritted disc and may therefore allow water to

bypass the filter paper. Supporting this hypothesis was the observation that the filtration process at the USUHS Water Lab took approximately 30 seconds to filter 50 ml of sample water, whereas the filtration process at MBAC took approximately one hour to filter 200 ml of sample water (and produced detectable quantities of DNA), using filter paper with the same specifications. While differences in vacuum pressure can account for some differences in filtration time, it is highly unlikely they explain such a dramatic difference. Thus, it is a real possibility that significant quantities of water were bypassing the filter paper, therefore reducing the quantity of bacteria adhering to the filter paper and reducing the likelihood of sufficient quantities of DNA remaining to surpass the threshold required for detection via traditional PCR.

Although running short quality-assurance experiments to test the hypotheses presented above for the non-detected DNA would be fairly simple, due to time constraints, it was not an option for the current study.

Because there were only six viable DNA samples, there was an insufficient sample size to obtain sufficient statistical power for analysis of the PCR data. One of the six viable samples (Site A) produced the nonsensical result that suggested there was greater than 100% human-associated Bacteroides present. Clearly, this result is due either to an experimental error or because of cross-sensitivity issues, as described above, with the assays that were used.

Future Research

Future work should increase the number samples analyzed via PCR in order to determine a more reliable estimate of the proportion of human-associated fecal

contamination in Rock Creek. In future studies, based on the lessons learned from this study, the following quality control measures are recommended:

- Conduct a trial early on in the study using various sample water quantities (collected from the source water to be tested) to confirm the minimum quantity required to obtain detectable DNA.
- Conduct a sample trial to confirm the selected primers produce observable DNA via traditional PCR.
- Utilize a two-piece Buchner funnel vacuum flask apparatus to ensure the entire quantity of sample water pass through the filter paper.
- Test rinse water for residual sodium hypochlorite bleach using DPD (diethyl paraphenylene diamine) indicator test.

While there is a "lack of widely accepted and standardized techniques for the MST methods, raising questions about the reproducibility of results both within and across laboratories" (51), the U.S. EPA is currently conducting a large multi-laboratory study seeking to develop a standardized method for qPCR use in microbial water quality management (43).

For additional consideration, Odagiri et al (31) recommend that BacCan, a dog-associated assay, can be used to discriminate between dog fecal contamination which may be detected by BacHum, as BacCan is 90% sensitive and 96% specific for canine fecal contamination and has not been shown to be cross-reactive human fecal contamination. This assay would be a useful adjunct in domestic/recreation areas such as Rock Creek.

A future study in the Rock Creek watershed could investigate in more depth the apparent trend of decreasing levels of E. coli following successive significant rainfalls. In such a study, it would be beneficial to expand the water sample collection period from May through September in order to increase the likelihood of obtaining a sufficient sample size of significant, successive rainfalls to determine whether a statistically significant trend exists. Sample collection days should be rain-event dependent, i.e. plan to collect samples for two to three days directly following any substantial rainfall. Using MST techniques, it would be of interest to determine whether human-associated fecal contamination also follows this trend. The incorporation of sewer system data would provide insight and further clarification as to the source (sewer versus run-off) of fecal contamination.

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APPENDIX A: Acronyms

AFRRI - Armed Forces Radiobiology Research Institute

BLAST - Basic Local Alignment Search Tool

bp - base pair

CFR - Code of Federal Regulations

CFU - colony forming units

COMAR - Code of Maryland Regulations

CSO - combined sewer overflow

CWA - Clean Water Act

D.C. - District of Columbia

DI - deionized

DNA – deoxyribonucleic acid

dNTPs - 2'-deoxynucleotide 5'triphosphates

DPD - diethyl paraphenylene diamine

FIB - fecal indicator bacteria

FWPCA - Federal Water Pollution Control Act

GIS - Geographic Information System

GMU - George Mason University

HDPE - High-density polyethylene

IBI - Index of Biotic Integrity

LIM - library independent methods

MBAC - Microbiome Analysis Center

MD - Maryland

MDLRC - Maryland's Lower Rock Creek

MOE - margin of error

MPN - most probable number

MST - microbial source tracking

NCEI - National Centers for Environmental Information

NPDES - National Pollutant Discharge Elimination System

NTU - Nephelometric Turbidity Units

p - Significance Level

PCR - polymerase chain reaction

PFGE - pulsed-field gel electrophoresis

qPCR - quantitative polymerase chain reaction

RC - Rock Creek

RNA - ribonucleic acid

SD - standard deviations

SFIB - standard fecal indicator bacteria

SPA - Special Protection Area

SSO - sanitary sewer overflow

TDS - Total Dissolved Solids

TMDL - Total Maximum Daily Limit

U.S. - United States

U.S. EPA - United States Environmental Protection Agency

USGS - United States Geological Survey

USUHS - Uniformed Services University of the Health Sciences

WQS - water quality standards

WSSC - Washington Suburban Sanitary Commission

APPENDIX B: Microbial Source Tracking Methods

Table B1. Advantages and Disadvantages of MST Methods

Method	Advantages	Disadvantages
PFGE	- Highly reproducible - Sensitive of minute genetic differences - May discriminate isolate from multiple host groups	- Labor-intensive - Requires cultivation of target organism - Requires specialized training of personnel - Requires reference library - Libraries may be geographically specific - Libraries may be temporally specific
Ribotyping	- Highly reproducible - Can be automated - May discriminate isolate from multiple host groups	- Labor-intensive (unless automated system used) - Requires cultivation of target organism - Requires reference library - Requires specialized training of personnel - Variations in methodology - Libraries may be geographically specific - Libraries may be temporally specific
rep-PCR	- Highly reproducible - Rapid; easy to perform - Requires limited training - May discriminate isolate from multiple host groups	- Requires reference library - Requires cultivation of target organism - Libraries may be geographically specific - Libraries may be temporally specific
Antibiotic Resistance	- Rapid; easy to perform - Requires limited training - May discriminate isolate from multiple host groups	- Require reference library - Requires cultivation of target organism - Libraries geographically specific - Libraries temporally specific - Variations in methods in different studies
Carbon Utilization	- Rapid; easy to perform - Requires limited training	- Require reference library - Requires cultivation of target organism - Libraries geographically specific - Libraries temporally specific - Variations in methods in different studies - Results often inconsistent
Bacteriophage (F+ coliphage)	- Distinguishes human from animals - Subtypes are stable characteristics - Easy to perform - Does not require a reference library	- Requires cultivation of coliphages - Sub-types do not exhibit absolute host specificity - Low in numbers in some environments
Host-specific bacterial PCR*	- Host specific - Does not require cultivation of target organism - Rapid; easy to perform - Does not require a reference library - Can identify multiple sources from same sample	- Little is known about survival and distribution in water systems - Primers currently not available for all relevant hosts
Host-specific viral PCR	- Host specific - Does not require cultivation of target organism - Easy to perform - Does not require reference library	- Often present in low numbers; requires large sample size - Not always present even when humans present

*Technique used in the current study

Reference: Tetra Tech Inc. and Herrera Environmental Consultants (51)

Table B2. Comparison of MST Methods for Use in TMDL Studies

Method	Library	Culture	Common Targets	Human/ Animal Sources Identified	Accuracy	Cost	Time Required
PFGE	Yes	Yes	*E. coli*	All species/ groups	High with large library	$100/isolate (e.g., 100 isolates/site)	2-4 days
Ribotyping	Yes	Yes	*E. coli*	All species/ groups	High with large library	Similar to PFGE	1-3 days
Rep-PCR	Yes	Yes	*E. coli*	All species/ groups	High with large library	Similar to PFGE	1 day
Antibiotic Resistance	Yes	Yes	*E. coli* Fecal enterococci Fecal streptococci	All species/ groups	Moderate with large library	Lower than PFGE if library is developed	4-5 days
Carbon Utilization	Yes	Yes	Enterococcus	All species/ groups	Moderate with large library	Lower than PFGE if library is developed	2-5 days
Bacterio-phage	No	Yes	F+ coliphage	Human, animals	Low-High depending on source/ experience	Low (<$100 /sample)	1-3 days
Viral PCR and qPCR	No	No	Human enterovirus/ polyomavirus, bovine enteroviruses, pig teschoviruses	Human, cow, pig	Moderate-High	$400/source/ sample	6-8 hrs (1-3 hrs, qPCR)
Bacterial PCR and qPCR*	No	No	Bacteroides, Enterococcus	Human, ruminants, cow, horse, dog, pig	Moderate-High	$400-600/ source/ sample	6-8 hrs (1-3 hrs, qPCR)

*Technique used in the current study

Reference: Tetra Tech Inc. and Herrera Environmental Consultants (51)

APPENDIX C: Water Sampling Authorization

June 10, 2016

Lt(N) Jonathan Honey
Uniformed Services University of the Health Sciences
4301 Jones Bridge Rd
Bethesda, MD 20814

Permission is hereby granted for Jonathan Honey to conduct water quality monitoring activities in Rock Creek as part of his thesis research in the following M-NCPPC Parks weekly from June – August 2016:

- Agricultural History Farm Park
- Rock Creek SVU 7
- Rock Creek SVU 5
- Rock Creek SVU 1

Please note that this permission covers only M-NCPPC parkland. Monitoring on private land or within other jurisdictions requires separate permission. Researchers must carry a copy of this letter at all times when conducting monitoring activities.

Matthew Harper
Principal Natural Resources Specialist
Park Planning and Stewardship Division
301-650-4383

PC: Jai Cole Mike Jones John Boyd Mohammed Turay
Park Police

Figure C1. Water Sampling Authorization Letter

APPENDIX D: Traditional PCR Gel Electrophoresis Images

Table D1. Traditional PCR Lane Assignment Details (Figure D1)

Lanes (L to R)	Sample (Date)	Dilution	Primers
1	Site A (7 Feb 2017)	1:5	GenBac
2	Site B (7 Feb 2017)	1:5	GenBac
3	Site C (7 Feb 2017)	1:5	GenBac
4	Site D (7 Feb 2017)	1:5	GenBac
5	Site E (7 Feb 2017)	1:5	GenBac
6	Site F (7 Feb 2017)	1:5	GenBac
7	Human stool	1:1	GenBac
8	Human stool	1:1	GenBac
9	E. coli	N/A	GenBac
10	B. frag	N/A	GenBac
11	B. thet	N/A	GenBac
12	Negative Control	N/A	GenBac
13	Negative Control	N/A	GenBac
14	E. coli	N/A	27f and 355r
15	B. frag	N/A	27f and 355r
16	Negative Control	N/A	27f and 355r, GenBac
17	Site A (7 Feb 2017)	1:5	Hf183/708r
18	Site B (7 Feb 2017)	1:5	Hf183/708r
19	Site C (7 Feb 2017)	1:5	Hf183/708r
20	Site D (7 Feb 2017)	1:5	Hf183/708r
21	Site E (7 Feb 2017)	1:5	Hf183/708r
22	Site F (7 Feb 2017)	1:5	Hf183/708r
23	Human stool	1:1	Hf183/708r
24	Human stool	1:1	Hf183/708r
25	E. coli	N/A	Hf183/708r
26	B. frag	N/A	Hf183/708r
27	B. thet	N/A	Hf183/708r
28	Negative Control	N/A	Hf183/708r
29	Negative Control	N/A	Hf183/708r
30	E. coli	N/A	Hf183/708r
31	B. frag	N/A	Hf183/708r
32	Negative Control	N/A	27f and 355r, Hf183/708r

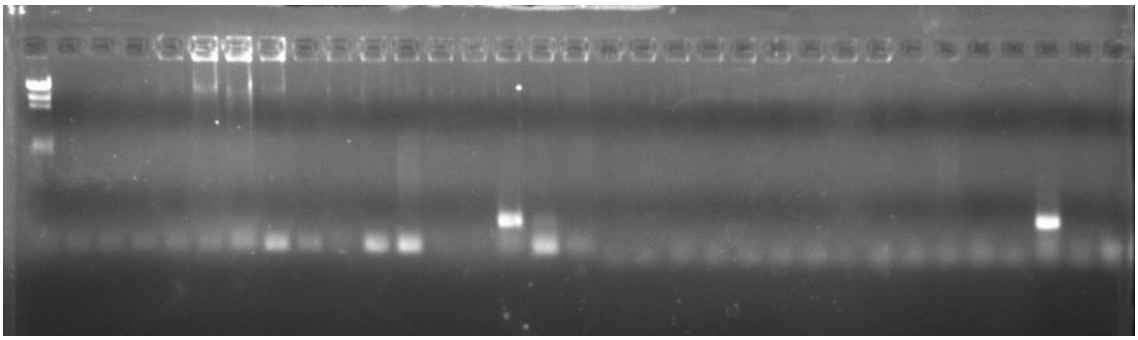

Figure D1. Agarose Gel Electrophoresis of DNA Products, Run 1
Image ID: RC_021617_4s, Duration: 30 minutes, 4 second gel photo exposure

Table D2. Traditional PCR Lane Assignment Details (Figure D2)

Lanes (L to R)	Sample (Date)	Dilution	Primers
1	Site A (7 Feb 2017)	1:1	L27f and 355r
2	Site B (7 Feb 2017)	1:1	L27f and 355r
3	Site C (7 Feb 2017)	1:1	L27f and 355r
4	Site D (7 Feb 2017)	1:1	L27f and 355r
5	Site E (7 Feb 2017)	1:1	L27f and 355r
6	Site F (7 Feb 2017)	1:1	L27f and 355r
7	N/A	N/A	L27f and 355r
8	N/A	N/A	L27f and 355r
9	N/A	N/A	L27f and 355r
10	Site A (7 Feb 2017)	1:5	L27f and 355r
11	Site B (7 Feb 2017)	1:5	L27f and 355r
12	Site C (7 Feb 2017)	1:5	L27f and 355r
13	Site D (7 Feb 2017)	1:5	L27f and 355r
14	Site E (7 Feb 2017)	1:5	L27f and 355r
15	Site F (7 Feb 2017)	1:5	L27f and 355r
16	N/A	N/A	L27f and 355r
17	N/A	N/A	L27f and 355r
18	N/A	N/A	L27f and 355r
19	E. coli (positive control)	1:10	L27f and 355r
20	B. fragilis (positive control)	1:10	L27f and 355r
21	negative control	N/A	L27f and 355r
Run duration: 30 minutes			

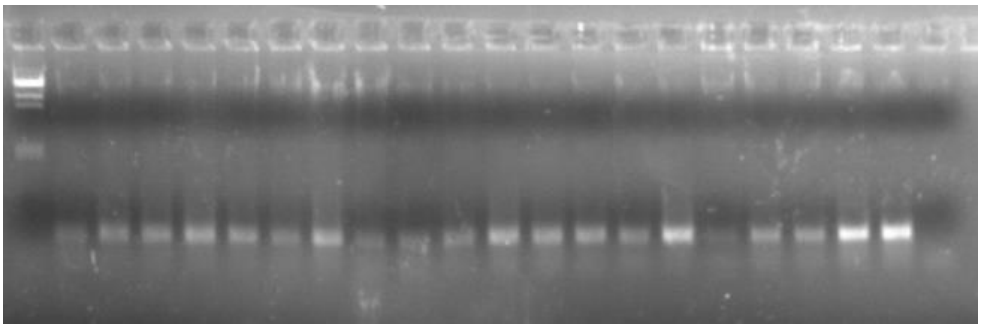

Figure D2. Agarose Gel Electrophoresis of DNA Products, Run 2
Image ID: Rock_Creek_021317

Table D3. Traditional PCR Lane Assignment Details (Figure D3)

Lanes (L to R)	Sample (Date)	Dilution	Primers
1	Site A (7 Feb 2017)		HF183, H241R
2	Site B (7 Feb 2017)		HF183, H241R
3	Site C (7 Feb 2017)		HF183, H241R
4	Site D (7 Feb 2017)		HF183, H241R
5	Site E (7 Feb 2017)		HF183, H241R
6	Site F (7 Feb 2017)		HF183, H241R
7			HF183, H241R
8			HF183, H241R
1	Site A (7 Feb 2017)		H160F, H241R
2	Site B (7 Feb 2017)		H160F, H241R
3	Site C (7 Feb 2017)		H160F, H241R
4	Site D (7 Feb 2017)		H160F, H241R
5	Site E (7 Feb 2017)		H160F, H241R
6	Site F (7 Feb 2017)		H160F, H241R
7			H160F, H241R
8			H160F, H241R
1	Site A (7 Feb 2017)		H193p, 355R
2	Site B (7 Feb 2017)		H193p, 355R
3	Site C (7 Feb 2017)		H193p, 355R
4	Site D (7 Feb 2017)		H193p, 355R
5	Site E (7 Feb 2017)		H193p, 355R
6	Site F (7 Feb 2017)		H193p, 355R
7			H193p, 355R
8	negative control	N/A	H193p, 355R

Figure D3. Agarose Gel Electrophoresis of DNA Products, Run 3

Table D4. Traditional PCR Lane Assignment Details (Figure D4)

Lanes (left to right)	Sample (Date)	Dilution	Primers
1	Site A (7 Feb 2017)		H193p, 355R
2	Site B (7 Feb 2017)		H193p, 355R
3	Site C (7 Feb 2017)		H193p, 355R
4	Site D (7 Feb 2017)		H193p, 355R
5	Site E (7 Feb 2017)		H193p, 355R
6	Site F (7 Feb 2017)		H193p, 355R
B.t	B. thet		H193p, 355R
B.f	B. frag		H193p, 355R
10	Site A (7 Feb 2017)		H193p, 355R
11	Site B (7 Feb 2017)		H193p, 355R
12	Site C (7 Feb 2017)		H193p, 355R
13	Site D (7 Feb 2017)		H193p, 355R
14	Site E (7 Feb 2017)		H193p, 355R
15	Site F (7 Feb 2017)		H193p, 355R
B.t	B. thet		H193p, 355R
B.f	B. frag		H193p, 355R
E.c	E. coli (positive control)		H193p, 355R
H	Human stool		H193p, 355R
N	Negative control		H193p, 355R
N	Negative control		H193p, 355R

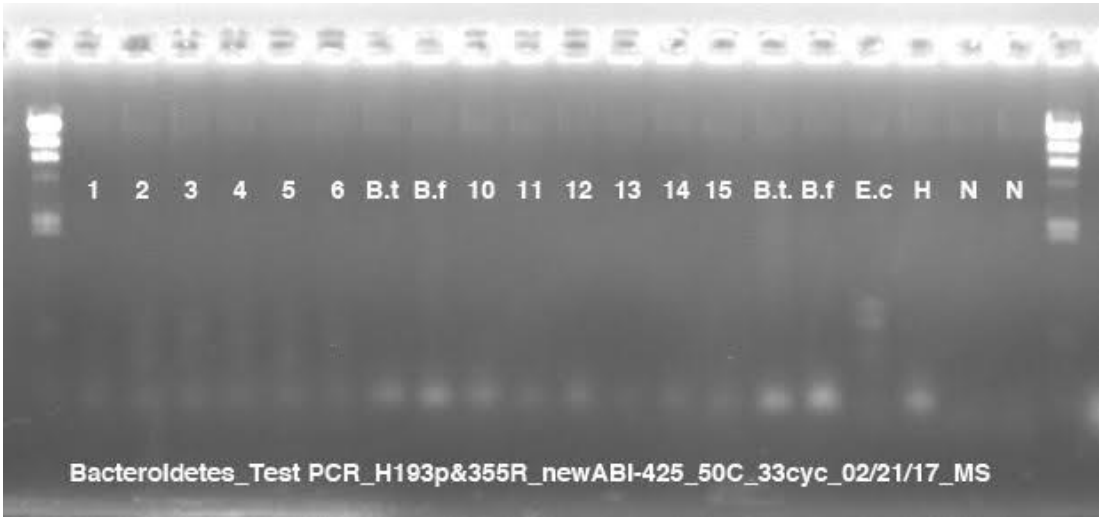

Figure D4. Agarose Gel Electrophoresis of DNA Products, Run 4

APPENDIX E: qPCR Protocol and Experimental Details

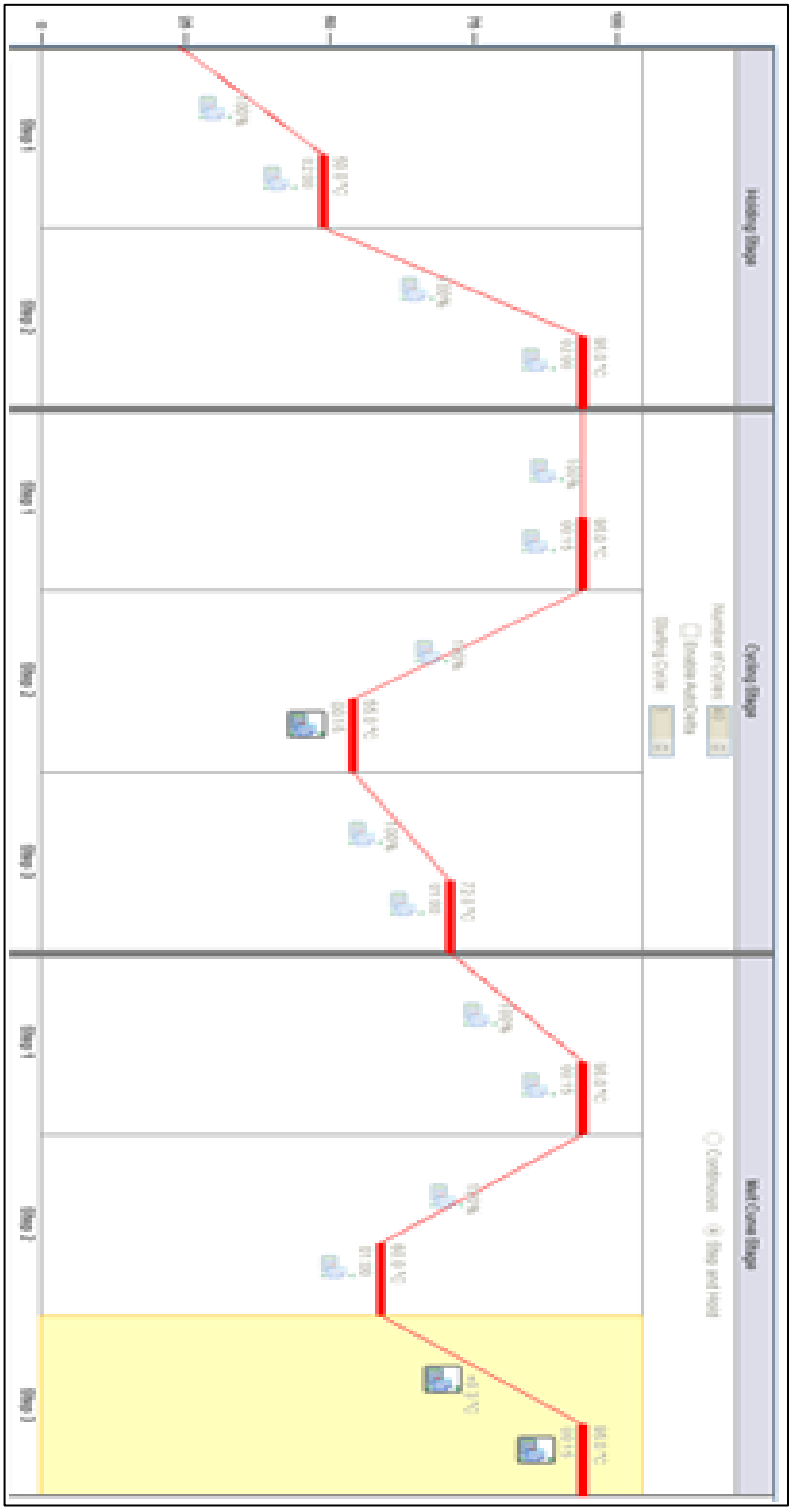

Figure E1. qPCR Protocol

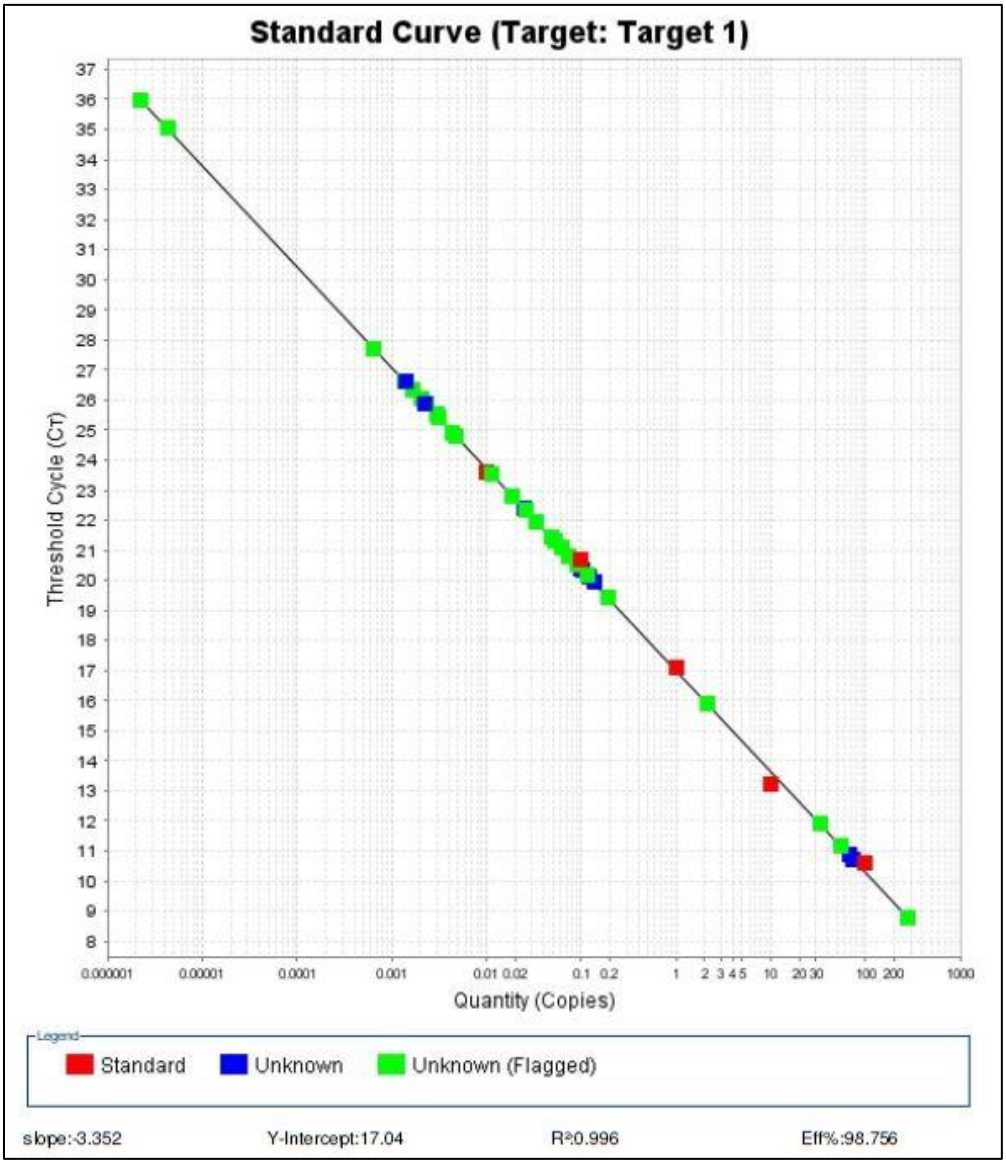

Figure E2. Standard Curve (Target 1, GenBac3)

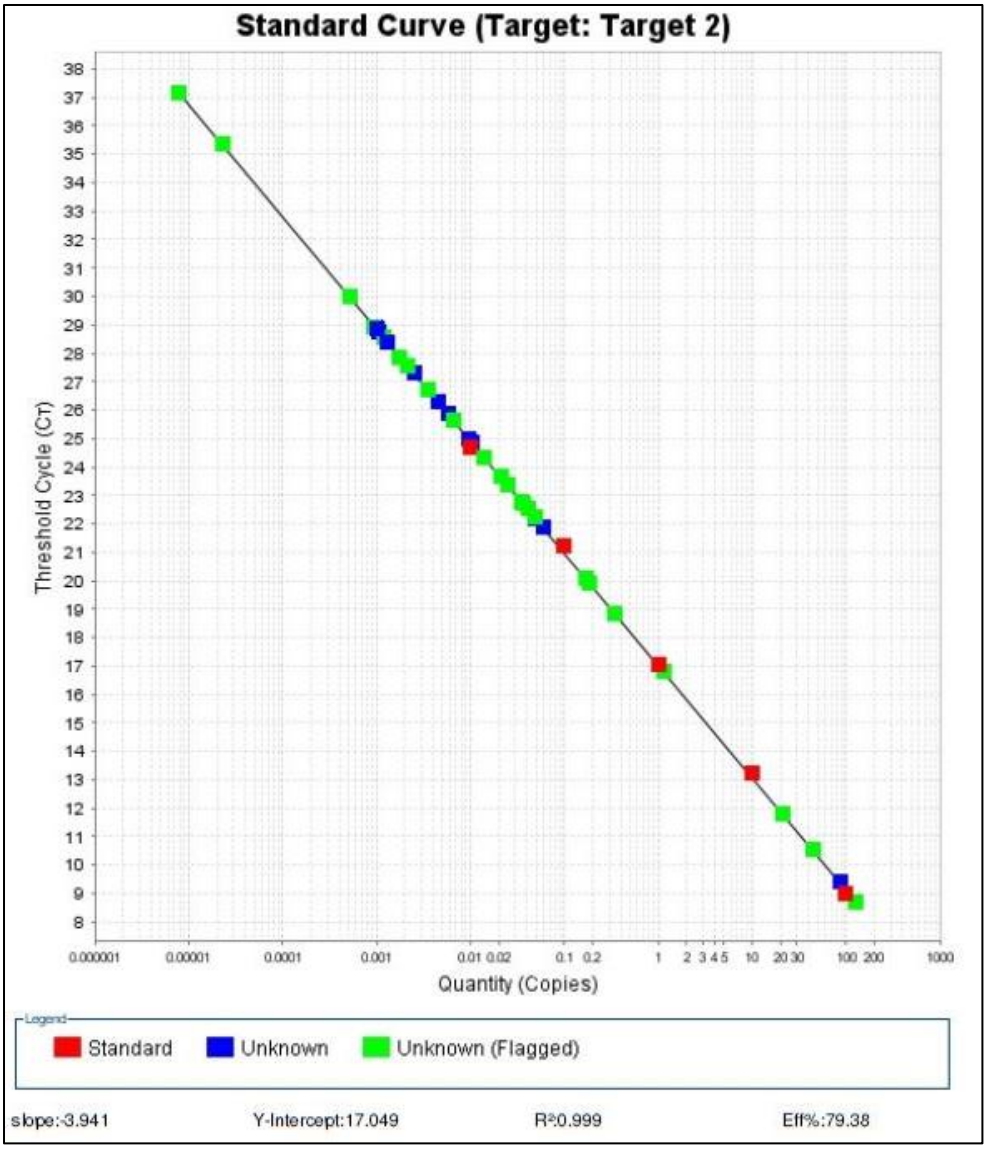

Figure E3. Standard Curve (Target 2, BacHum)

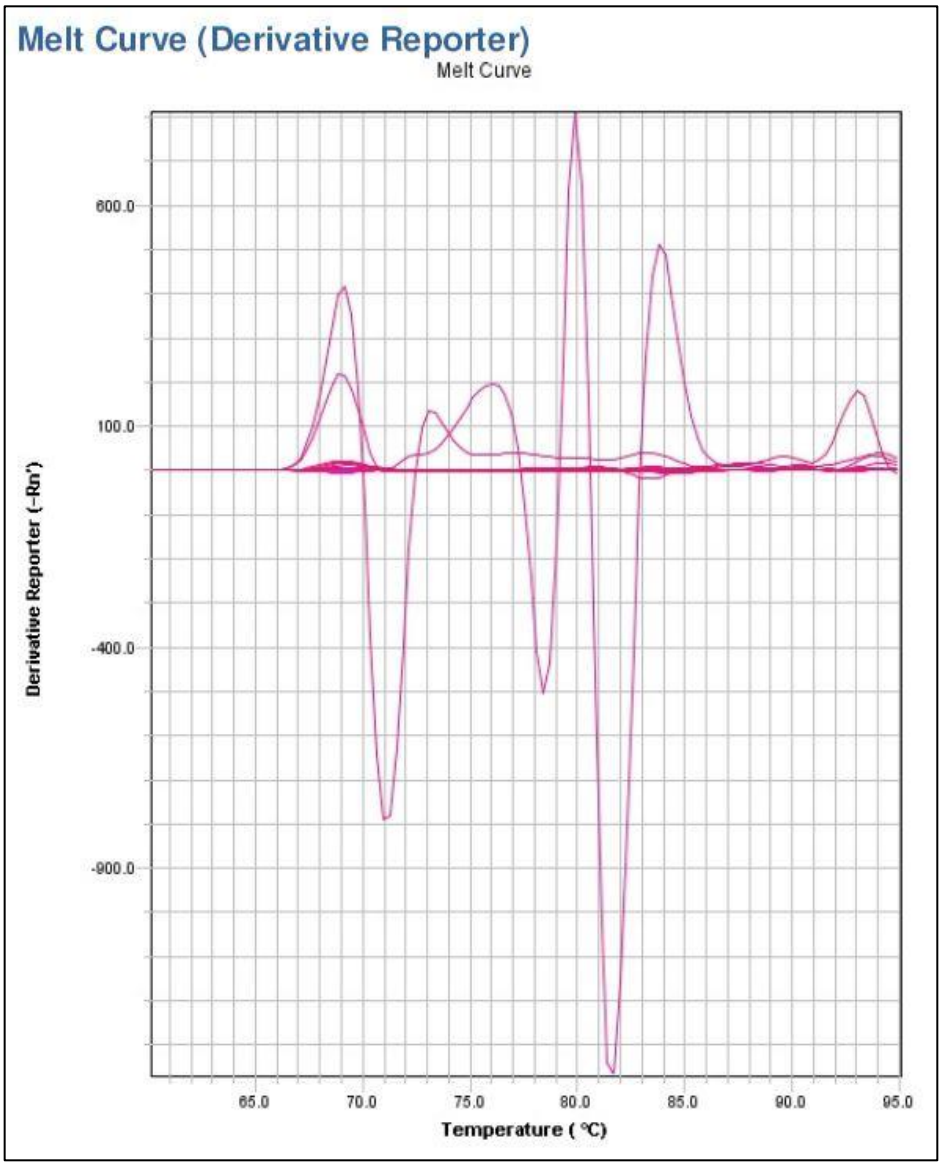

Figure E4. Melt Curve (Derivative Reporter)

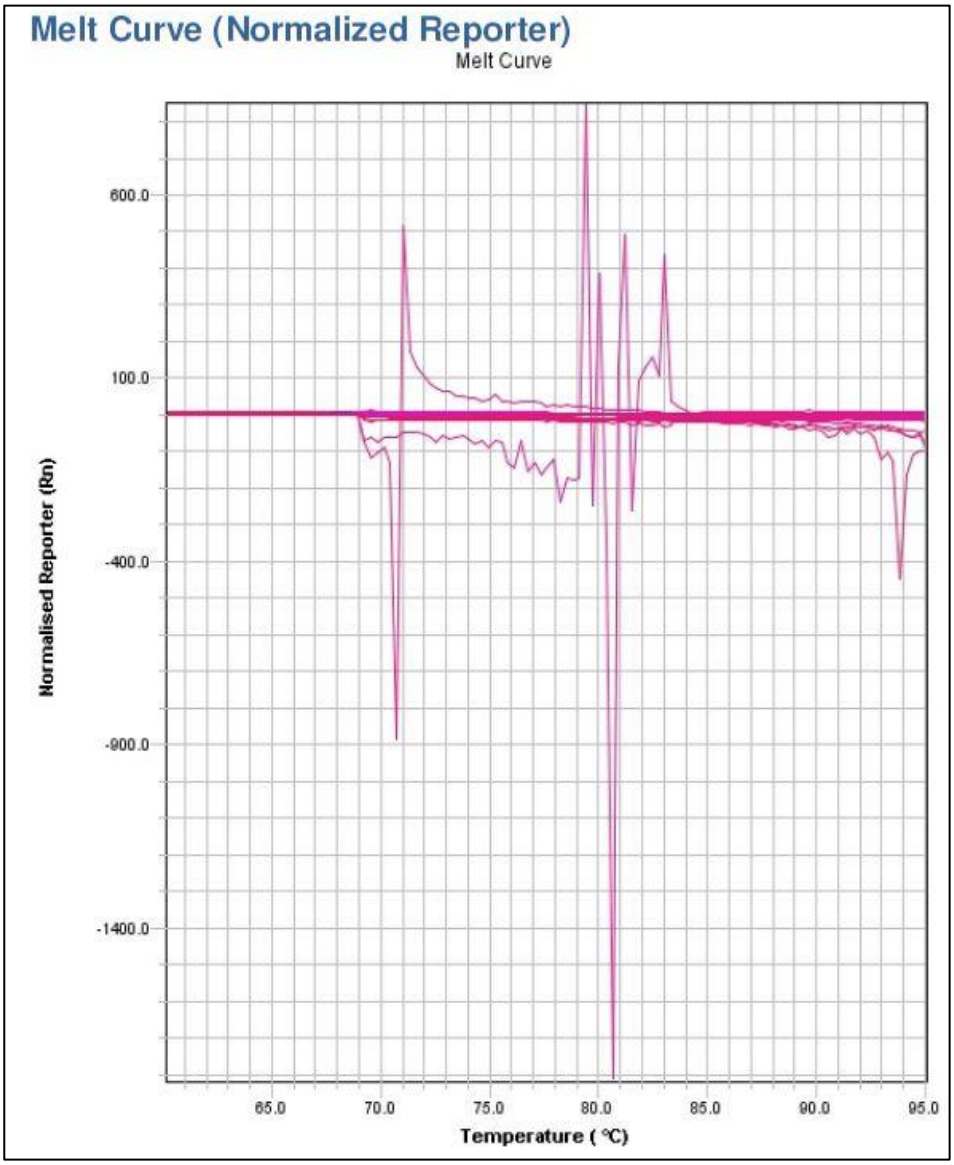

Figure E5. Melt Curve (Normalized Reporter)

Table E1. qPCR Results Table

Well	Sample Name	Target Name*	Task	Ct	Quant	Tm1	Tm2	Comments
A1	Standard1_B.Thetao	1	STANDARD	10.6	100.0	84.1		Standard1 B.thetaotaiomicron
B1	Standard2_B.Thetao	1	STANDARD	13.2	10.0	84.1		Standard2 B.thetaotaiomicron
C1	Standard3_B.Thetao	1	STANDARD	17.1	1.0	84.1		Standard3 B.thetaotaiomicron
D1	Standard4_B.Thetao	1	STANDARD	20.7	0.1	84.1		B.thetaotaiomicron
E1	Standard5_B.Thetao	1	STANDARD	23.6	0.0	69.0		B.thetaotaiomicron
A6	Unknown1_B.fragilis	1	UNKNOWN	8.8	286.4	83.9		Standard1 B.fragilis
B6	Unknown2_B.fragilis	1	UNKNOWN	11.2	55.7	83.8		Standard2 B.fragilis
C6	Unknown3_B.fragilis	1	UNKNOWN	11.9	34.0	83.8		Standard3 B.fragilis
D6	Unknown4_B.fragilis	1	UNKNOWN	15.9	2.2	83.8		B.fragilis
E6	Unknown5_B.fragilis	1	UNKNOWN	19.4	0.2	68.7		B.fragilis
A2	Sample 1	1	UNKNOWN	25.9	0.0	83.0		Standard1
A4	Sample 1	1	UNKNOWN	26.6	0.0	83.3		Standard1
B2	Sample 2	1	UNKNOWN	25.5	0.0	83.0		Standard2
B4	Sample 2	1	UNKNOWN	26.6	0.0	83.2		Standard2
C2	Sample 3	1	UNKNOWN	24.9	0.0	83.2		Standard3
C4	Sample 3	1	UNKNOWN	26.0	0.0	83.3		Standard3
D2	Sample 4	1	UNKNOWN	26.3	0.0	83.2		
D4	Sample 4	1	UNKNOWN	27.7	0.0	83.3		
E2	Sample 5	1	UNKNOWN	25.4	0.0	83.2		
E4	Sample 5	1	UNKNOWN	26.6	0.0	83.3		
F2	Sample 6	1	UNKNOWN	24.8	0.0	83.6		
F4	Sample 6	1	UNKNOWN	25.9	0.0	83.8		
G2	Sample 7	1	UNKNOWN	19.9	0.1	83.8		Human Stool#73
G4	Sample 7	1	UNKNOWN	20.1	0.1	83.8		Human Stool#73
H2	Sample 8	1	UNKNOWN	22.4	0.0	83.8		Human Stool#74
H4	Sample 8	1	UNKNOWN	22.4	0.0	83.8		Human Stool#74
A3	Sample 9	1	UNKNOWN	36.0	0.0	83.8		E. coli_1:10dil
A5	Sample 9	1	UNKNOWN	35.0	0.0	83.8		E. coli_1:10dil
B3	Sample 10	1	UNKNOWN	20.2	0.1	83.3		
B5	Sample 10	1	UNKNOWN	21.1	0.1	83.6		
C3	Sample 11	1	UNKNOWN	22.8	0.0			
C5	Sample 11	1	UNKNOWN	23.6	0.0	83.2		
D3	Sample 12	1	UNKNOWN	20.5	0.1	83.0		
D5	Sample 12	1	UNKNOWN	21.3	0.1	83.6		
E3	Sample 13	1	UNKNOWN	20.8	0.1	83.2		
E5	Sample 13	1	UNKNOWN	22.0	0.0	83.6		
F3	Sample 14	1	UNKNOWN	21.5	0.0	81.7		
F5	Sample 14	1	UNKNOWN	22.4	0.0	82.9		
G3	Sample 15	1	UNKNOWN	20.4	0.1	84.4		
G5	Sample 15	1	UNKNOWN	20.3	0.1	84.2		
H3	Sample 16	1	UNKNOWN	10.9	67.9	69.0		B.thetaotaiomicron 1ul-nodil
H5	Sample 16	1	UNKNOWN	10.7	75.6	79.9		B.thetaotaiomicron 1ul-nodil
F1	Negative1	1	NTC	35.7		83.6		

G1	Negative2	1	NTC	34.9		69.0		
H1	Negative3	1	NTC	36.4		69.3		
F6	Negative4	1	NTC	U		68.7		
G6	Negative5	1	NTC	U		74.5		
H6	Negative6	1	NTC	37.0		69.2		
A7	Standard1_B.Thetao	2	STANDARD	9.0	100.0	83.6		Standard1B.thetaotaiomicron
B7	Standard2_B.Thetao	2	STANDARD	13.2	10.0	83.6		Standard2 B.thetaotaiomicron
C7	Standard3_B.Thetao	2	STANDARD	17.1	1.0	83.5	68.7	Standard3 B.thetaotaiomicron
D7	Standard4_B.Thetao	2	STANDARD	21.2	0.1	83.3	68.7	B.thetaotaiomicron
E7	Standard5_B.Thetao	2	STANDARD	24.7	0.0	68.7		B.thetaotaiomicron
A12	Unknown1_B.fragilis	2	UNKNOWN	8.7	129.4	83.6		Standard1 B.fragilis
B12	Unknown2_B.fragilis	2	UNKNOWN	10.5	45.4	83.5		Standard2 B.fragilis
C12	Unknown3_B.fragilis	2	UNKNOWN	11.8	21.3	83.5	69.0	Standard3 B.fragilis
D12	Unknown4_B.fragilis	2	UNKNOWN	16.8	1.2	83.5	68.9	B.fragilis
E12	Unknown5_B.fragilis	2	UNKNOWN	20.1	0.2	68.7		B.fragilis
A8	Sample 1	2	UNKNOWN	26.7	0.0	83.3	87.7	Standard1
A10	Sample 1	2	UNKNOWN	27.6	0.0	83.2	87.5	Standard1
B8	Sample 2	2	UNKNOWN	28.4	0.0	83.3		Standard2
B10	Sample 2	2	UNKNOWN	28.9	0.0	83.3		Standard2
C8	Sample 3	2	UNKNOWN	27.3	0.0	83.2		Standard3
C10	Sample 3	2	UNKNOWN	27.9	0.0	83.3	68.9	Standard3
D8	Sample 4	2	UNKNOWN	28.9	0.0	83.3	68.6	
D10	Sample 4	2	UNKNOWN	30.0	0.0	83.5		
E8	Sample 5	2	UNKNOWN	28.6	0.0	83.3	68.6	
E10	Sample 5	2	UNKNOWN	28.9	0.0	83.5		
F8	Sample 6	2	UNKNOWN	28.8	0.0	83.8		
F10	Sample 6	2	UNKNOWN	28.6	0.0	83.8		
G8	Sample 7	2	UNKNOWN	26.3	0.0	83.8		Human Stool#73
G10	Sample 7	2	UNKNOWN	25.9	0.0	83.8		Human Stool#73
H8	Sample 8	2	UNKNOWN	24.9	0.0	69.2		Human Stool#74
H10	Sample 8	2	UNKNOWN	25.0	0.0	93.8		Human Stool#74
A9	Sample 9	2	UNKNOWN	37.2	0.0	83.6		E. coli_1:10dil
A11	Sample 9	2	UNKNOWN	35.3	0.0	83.8		E. coli_1:10dil
B9	Sample 10	2	UNKNOWN	18.9	0.3	83.6	68.9	
B11	Sample 10	2	UNKNOWN	19.9	0.2	83.8	69.0	
C9	Sample 11	2	UNKNOWN	24.3	0.0	83.0	69.0	
C11	Sample 11	2	UNKNOWN	25.6	0.0	83.5		
D9	Sample 12	2	UNKNOWN	22.2	0.0	83.6	69.0	
D11	Sample 12	2	UNKNOWN	22.7	0.0	84.1	69.2	
E9	Sample 13	2	UNKNOWN	22.5	0.0	83.8		
E11	Sample 13	2	UNKNOWN	23.4	0.0	84.1		
F9	Sample 14	2	UNKNOWN	22.8	0.0	68.7		
F11	Sample 14	2	UNKNOWN	23.7	0.0	83.0	69.0	
G9	Sample 15	2	UNKNOWN	22.2	0.0	68.7		
G11	Sample 15	2	UNKNOWN	21.9	0.1	83.6		
H9	Sample 16	2	UNKNOWN	U		94.1		B.thetaotaiomicron 1ul-nodil

H11	Sample 16	2	UNKNOWN	9.4	87.3	69.0		B.thetaotaiomicron 1ul-nodil
F7	Negative1	2	NTC	U		68.9		
G7	Negative2	2	NTC	U		61.6		
H7	Negative3	2	NTC	U		88.0		
F12	Negative4	2	NTC	U		68.7		
G12	Negative5	2	NTC	U		61.6		
H12	Negative6	2	NTC	U		93.0		

*Target 1 – GenBac3, Target 2 – BacHum

Table E2. qPCR Quality Control Summary

QC Summary

Total Wells	96	Processed Wells	96	Targets Used	2
Well Setup	96	Flagged Wells	53	Samples Used	16

Flag	Name	Frequency	Locations
AMPNC	Amplification in negative control	1	G1
BADROX	Bad passive reference signal	0	
BLFAIL	Baseline algorithm failed	0	
CTFAIL	Cт algorithm failed	0	
EXPFAIL	Exponential algorithm failed	0	
HIGHSD	High standard deviation in replicate group	46	A3, A5, A6, A8, A9, A10, A11, A12, B2, B3, B4, B5, B6, B9, B11, B12, C2, C3, C4, C5, C6, C9, C11, C12, D2, D3, D4, D5, D6, D8, D10, D12, E2, E3, E4, E5, E6, E9, E11, E12, F2, F3, F4, F5, F9, F11
MTP	Multiple Tm peaks	15	A8, A10, B9, B11, C7, C9, C10, C12, D7, D8, D9, D11, D12, E8, F11
NOAMP	No amplification	0	
NOISE	Noise higher than others in plate	0	
NOSIGNAL	No signal in well	0	
OFFSCALE	Fluorescence is offscale	0	
OUTLIERRG	Outlier in replicate group	0	
SPIKE	Noise spikes	0	
THOLDFAIL	Thresholding algorithm failed	0	

APPENDIX F: Standard Water Quality Parameter Results by Site

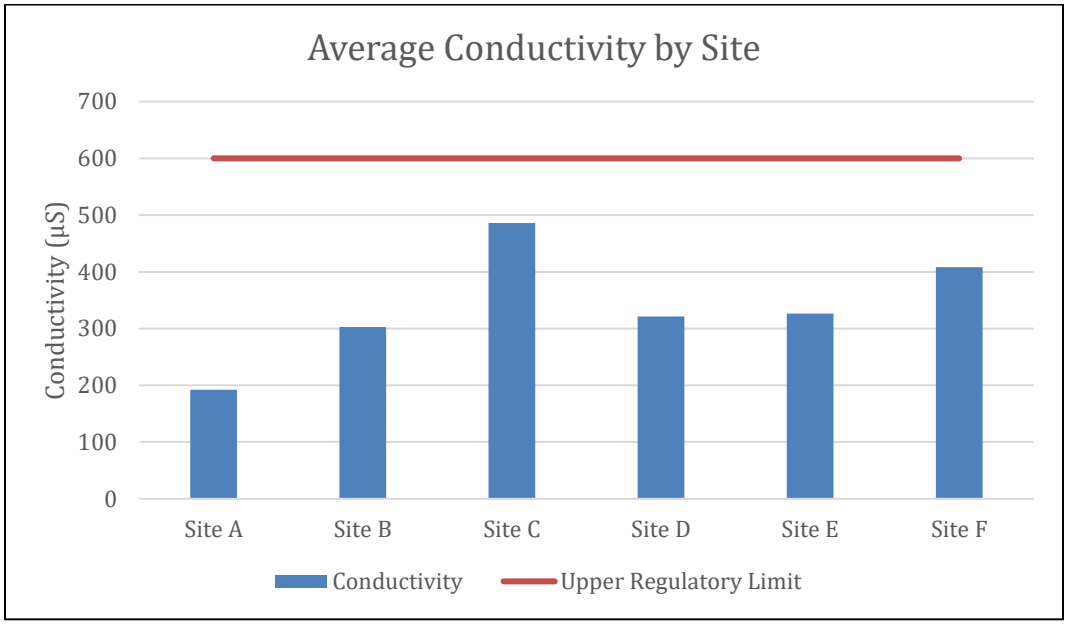

Figure F1. Average Conductivity by Site

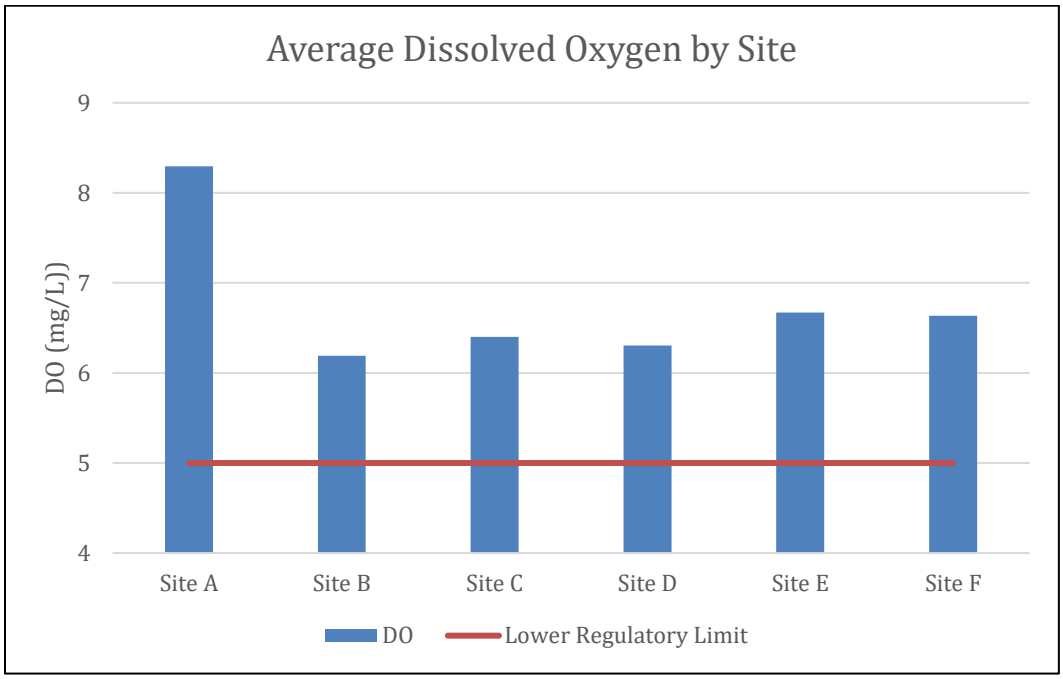

Figure F2. Average DO by Site

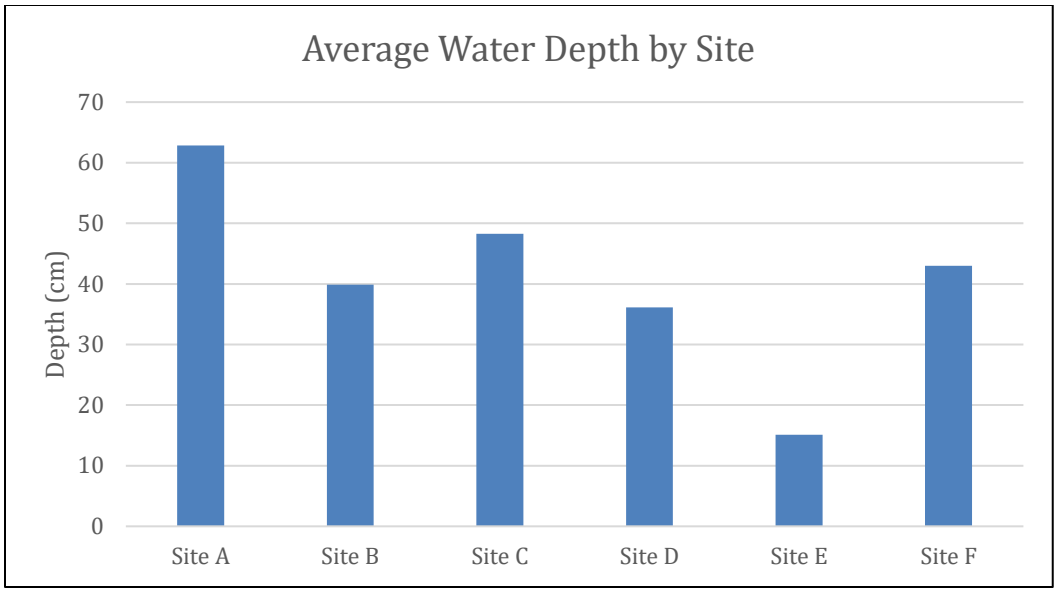

Figure F3. Average Water Depth by Site

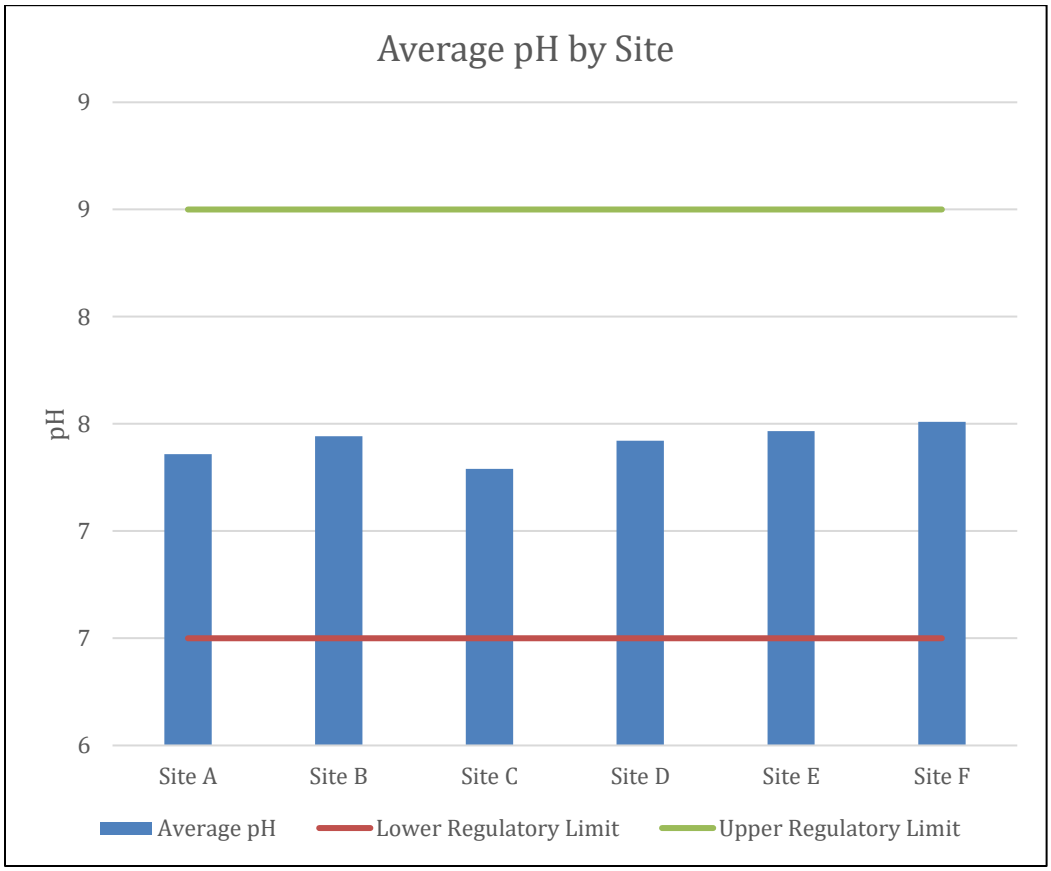

Figure F4. Average pH by Site

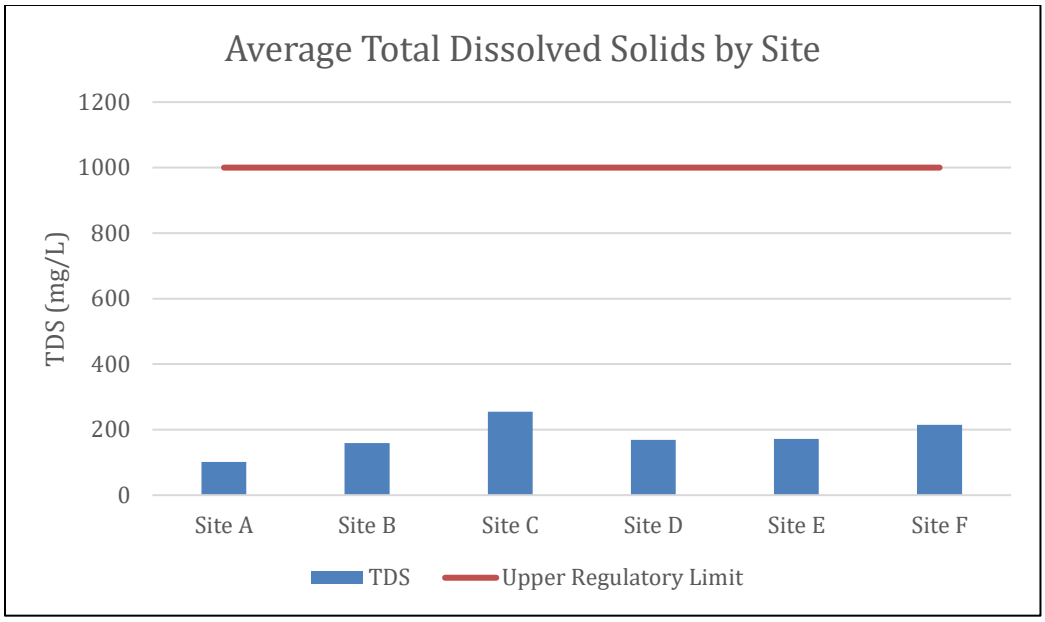

Figure F5. Average TDS by Site

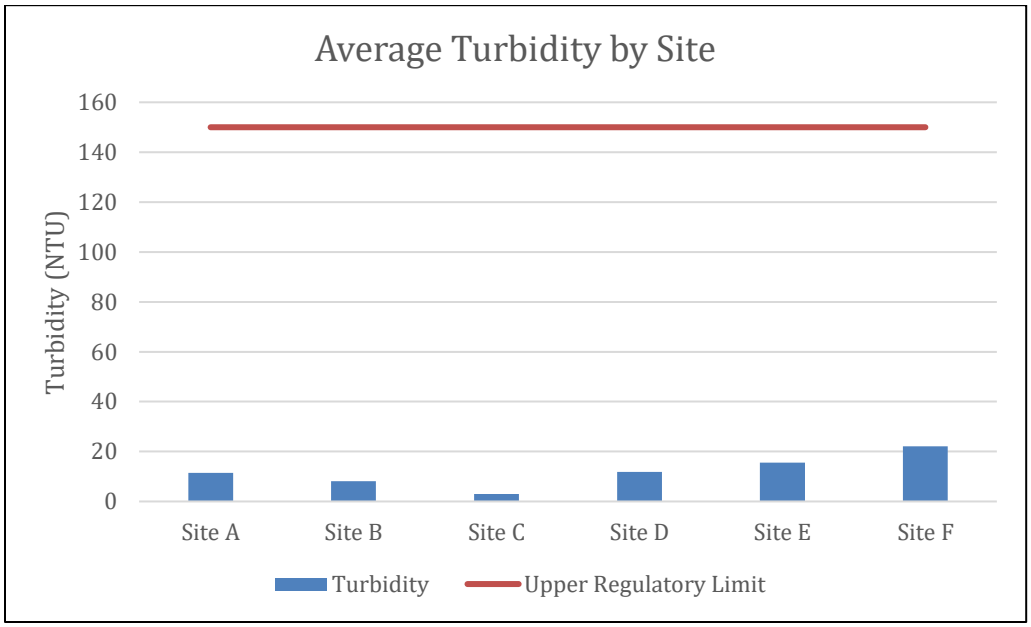

Figure F6. Average Turbidity by Site

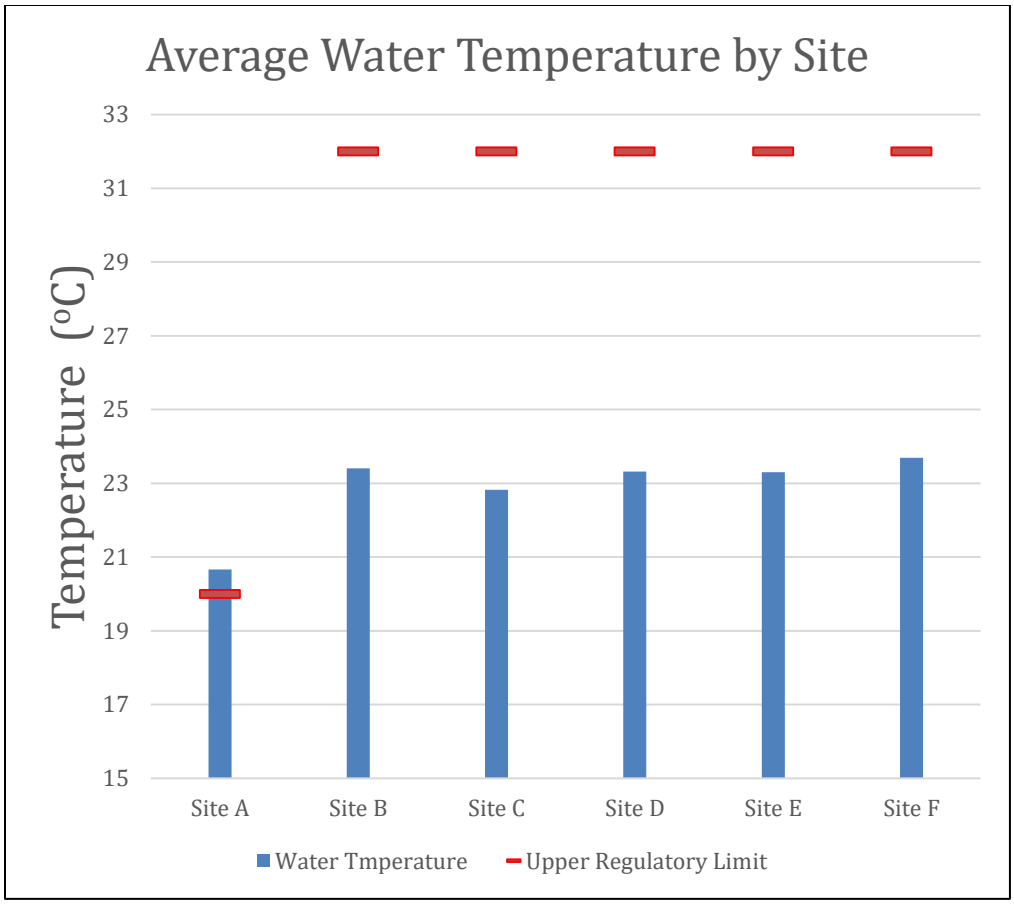

Figure F7. Average Water Temperature by Site
*Site A falls under Designated Use Class 3 and therefore has differing temperature regulations (Table 12)

APPENDIX G: Raw Data

Table G1. Raw Data (Discharge, Rainfall, Water Temperature, TDS)

	Avg Discharge (ft^3/sec)	Rainfall (inches) by site						Water Temp (°C)						TDS (mg/L)					
		A	B	C	D	E	F	A	B	C	D	E	F	A	B	C	D	E	F
16-Jul-16	48.0	0.12	0.04	0.12	0.12	0.12	0.3												
17-Jul-16	30.5	0.01	0	0.01	0.01	0.01	0												
18-Jul-16	36.8	0.34	0.12	0.39	0.39	0.39	0.38												
19-Jul-16	93.7	0.17	0.21	0.97	0.97	0.97	0.94												
20-Jul-16	209.9	0.12	0.04	0.01	0.01	0.01	0	21.4	24.4	23.2	23.5	23.2	23.3	104.9	211.3	151.9	168.1	149.1	111.5
21-Jul-16	28.4	0	0	0	0	0	0												
22-Jul-16	21.6	0	0	0	0	0	0	20.9	24.2	22.7	23.7	23.7	24.0	106.4	184.1	265.4	205.3	215.5	232.2
23-Jul-16	19.8	0	0	0	0	0	0												
24-Jul-16	17.8	0.16	0	0.15	0.15	0.15	0.02												
25-Jul-16	19.6	0	0	0.01	0.01	0.01	0	22.9	25.7	24.5	25.9	25.8	26.5	106.4	190.6	330.2	217.3	220.9	315
26-Jul-16	16.1	0.03	0	0	0	0	0												
27-Jul-16	14.8	0.11	0	0	0	0	0	22.6	25.2	24.6	25.8	25.8	26.5	106.1	204.3	344	228.5	240.3	332
28-Jul-16	142.5	1.41	2.34	1.11	1.11	1.11	1.43												
29-Jul-16	458.1	0.85	0.7	0.49	0.49	0.49	0.74	22.0	27.0	24.7	25.6	24.8	25.2	75.21	138.1	85.94	104.8	69.77	86.3
30-Jul-16	222.1	1.81	1.24	0.83	0.83	0.83	0.84												
31-Jul-16	364.0	0	0.04	0.12	0.12	0.12	0.16												
01-Aug-16	141.9	0	0	0	0	0	0	21.4	25.8	24.3	25.2	25.2	25.3	90.75	104.5	145.7	107.2	109.9	121.3
02-Aug-16	99.7	0	0	0	0	0	0												
03-Aug-16	70.3	0	0	0	0	0	0	20.9	25.5	22.9	25.0	25.0	25.1	102.1	108.9	240.8	113.9	120.9	147.8
04-Aug-16	44.2	0	0	0	0	0	0												
05-Aug-16	33.8	0	0	0	0	0	0	20.0	25.0	22.2	23.9	23.7	23.6	104.4	126.7	296.6	139.9	149.5	193.1
06-Aug-16	32.5	0	0	0.08	0.08	0.08	0.09												
07-Aug-16	27.2	0.1	0	0	0	0	0												
08-Aug-16	21.4	0.01	0	0	0	0	0	20.0	23.9	21.7	23.2	23.1	23.8	104.2	144.9	329.8	168.8	181.1	238.8
09-Aug-16	20.6	0	0	0	0	0	0												
10-Aug-16	19.3	0.11	0	0	0	0	0	20.7	24.3	22.4	23.5	23.3	23.2	104.7	160.1	344.1	189	202.8	282.6
11-Aug-16	18.6	0	0	0	0	0	0												
12-Aug-16	16.9	0	0.08	0	0	0	0	22.7	25.4	24.3	25.5	25.6	25.9	105	166.3	358	198.2	211	306.3
13-Aug-16	20.2	0	0	0	0	0	0												
14-Aug-16	16.6	0.14	0.16	0.06	0.06	0.06	0.07												
15-Aug-16	80.9	2.39	1.61	0.69	0.69	0.69	0.92	22.8	25.1	24.5	25.5	25.8	26.5	104.2	191.8	237.2	194.3	180.4	303.2
16-Aug-16	226.5	0.03	0	0.01	0.01	0.01	0												
17-Aug-16	131.2	1.26	0.49	0.45	0.45	0.45	0.5	22.6	26.2	25.0	26.0	25.9	26.2	91.36	100.2	165.5	105.4	107	126
18-Aug-16	196.8	0	0.04	0.01	0.01	0.01	0												
19-Aug-16	83.5	0	0	0	0	0	0	21.5	25.8	23.7	25.3	25.1	25.4	94.08	99.75	173	105.9	107.5	128.5
20-Aug-16	50.8	0	0	0	0	0	0												
21-Aug-16	186.7	0.12	0.38	0.96	0.96	0.96	1.41												
22-Aug-16	80.0	0	0	0	0	0	0												
23-Aug-16	32.5	0	0	0	0	0	0	18.9	23.3	20.8	22.4	22.2	22.4	103.9	120.3	200.9	131.2	137.7	152.6
24-Aug-16	25.3	0	0	0	0	0	0												
25-Aug-16	21.4	0	0	0	0	0	0												
26-Aug-16	20.6	0	0	0	0	0	0												
27-Aug-16	18.1	0.19	0	0	0	0	0												
28-Aug-16	15.6	0.01	0	0	0	0	0												
29-Aug-16	14.7	0	0	0	0	0	0												
30-Aug-16	13.9	0	0	0	0	0	0	20.9	23.7	22.3	23.6	23.5	24.2	104	161.8	352.6	189.9	201.5	286.4
31-Aug-16	12.9	0	0	0	0	0	0												
01-Sep-16	45.7	0.11	0.12	0.95	0.95	0.95	0.53												
02-Sep-16	64.8	0.01	0.04	0.01	0.01	0.01	0	19.3	21.7	21.1	21.8	21.8	22.7	105.7	195.9	201.8	172.9	155.7	115.4
03-Sep-16	15.0	0.26	0	0	0	0	0												
04-Sep-16	11.8	0	0	0	0	0	0												
05-Sep-16	10.7	0.04	0	0	0	0	0												
06-Sep-16	10.0	0	0	0	0	0	0	18.5	20.7	19.6	20.3	20.2	21.0	104.7	182.4	332	201.7	221	302
07-Sep-16	31.8	0.56	0.12	0.48	0.48	0.48	0.27												
08-Sep-16	67.7	0.04	0	0.01	0.01	0.01	0												
09-Sep-16	24.4	0	0	0	0	0	0	21.6	24.8	23.8	24.5	24.4	24.5	93.63	158.4	219	191.2	204.5	175.8
10-Sep-16	16.7	0	0	0	0	0	0												
11-Sep-16	14.0	0	0	0	0	0	0												
12-Sep-16	10.7	0	0	0	0	0	0												
13-Sep-16	9.9	0	0	0	0	0	0	18.7	20.7	20.2	20.8	21.0	21.9	104	179.5	328.4	188.4	207.5	275.2
14-Sep-16	9.6	0	0	0	0	0	0												
15-Sep-16	8.3	0	0	0	0	0	0												
16-Sep-16	7.8	0.04	0	0	0	0	0												
17-Sep-16	7.8	0	0	0	0	0	0												
18-Sep-16	7.5	0.11	0	0	0	0	0												
19-Sep-16	26.5	0.32	0.24	0.29	0.29	0.29	0.39												
20-Sep-16	21.8	0	0	0	0	0	0	19.4	21.3	21.1	21.5	21.5	22.0	104.4	203.6	247.1	217.5	203.7	266.4
07-Feb-17								5.8	4.9	6.3	5.4	5.1	5.1	108.5	223.6	399.1	240.2	258.8	324.4

Table G2. Raw Data (Conductivity, Depth, DO)

	Avg Discharge	Conductivity (µS) by site						Depth (cm) by site						DO (mg/L) by site					
	(ft^3/sec)	A	B	C	D	E	F	A	B	C	D	E	F	A	B	C	D	E	F
16-Jul-16	48.0																		
17-Jul-16	30.5																		
18-Jul-16	36.8																		
19-Jul-16	93.7																		
20-Jul-16	209.9	210	423	304	337	298	223	58.5	41	53.5	20	16.5	58	8.28	6.65	7.34	6.01	6.26	6.2
21-Jul-16	28.4																		
22-Jul-16	21.6	213	368	532	411	431	464.6	58	40	57	28	17.5	23	8.18	6.23	6.52	6.3	6.62	6.42
23-Jul-16	19.8																		
24-Jul-16	17.8																		
25-Jul-16	19.6	213	382	661	435	442	629.7	54.9	22.4	49.5	26.8	10.2	35.5	7.74	5.58	5.98	5.65	5.31	6.25
26-Jul-16	16.1																		
27-Jul-16	14.8	212	409	688	458	481	664.3	70	22.0	49.5	26.0	7.0	31.0	7.98	5.65	6.3	5.74	6.43	6.13
28-Jul-16	142.5																		
29-Jul-16	458.1	150	276	172	209	140	172.3	56	68.0	70.0	60.0	71.0	100.0	7.8	7.22	7.65	6.58	6.86	6.43
30-Jul-16	222.1																		
31-Jul-16	364.0																		
01-Aug-16	141.9	181	209	293	215	220	242.6	48	62.0	50.5	66.0	26.0	71.0	8.18	7.42	6.71	6.97	7.07	6.98
02-Aug-16	99.7																		
03-Aug-16	70.3	204	218	482	228	242	295.6	61	48.0	46.0	49.0	18.0	55.0	8.12	7.01	6.61	6.75	7.02	7.08
04-Aug-16	44.2																		
05-Aug-16	33.8	208	254	593	280	299	386.1	65	45.0	52.0	40.0	9.0	38.0	8.49	6.47	7.15	6.64	7.15	7.15
06-Aug-16	32.5																		
07-Aug-16	27.2																		
08-Aug-16	21.4	208	290	659	336	362	477.6	66	39.0	57.0	33.5	10.0	34.0	8.65	6.16	6.77	6.57	7.17	6.93
09-Aug-16	20.6																		
10-Aug-16	19.3	209	320	688	378	405	565.1	73	39.0	50.0	37.0	10.0	31.0	8.64	6.19	7.24	6.69	7.42	7.31
11-Aug-16	18.6																		
12-Aug-16	16.9	192	303	653	362	385	559.3	73	37.0	43.0	27.0	10.0	31.0	7.88	5.67	6.29	6.11	6.81	6.75
13-Aug-16	20.2																		
14-Aug-16	16.6																		
15-Aug-16	80.9	190	350	433	356	329	553.2	75	32.0	42.0	30.0	9.5	44.0	7.56	5.17	5.57	5.25	5.54	6.54
16-Aug-16	226.5																		
17-Aug-16	131.2	167	183	302	193	195	229.9	48	63.0	43.0	63.0	24.0	63.5	7.84	7.14	6.31	6.79	6.89	6.76
18-Aug-16	196.8																		
19-Aug-16	83.5	171	182	316	193	196	234.5	48	50.0	48.0	55.0	16.0	54.0	7.97	7	6.51	6.77	6.91	6.84
20-Aug-16	50.8																		
21-Aug-16	186.7																		
22-Aug-16	80.0																		
23-Aug-16	32.5	190	220	367	239	251	278.5	62	39.0	47.0	36.0	11.5	41.0	8.65	6.3	6.63	6.72	7.13	6.88
24-Aug-16	25.3																		
25-Aug-16	21.4																		
26-Aug-16	20.6																		
27-Aug-16	18.1																		
28-Aug-16	15.6																		
29-Aug-16	14.7																		
30-Aug-16	13.9	190	295	644	347	368	522.3	75	33.0	47.0	27.0	9.0	29.0	9.18	5.45	6.24	6.25	7	6.64
31-Aug-16	12.9																		
01-Sep-16	45.7																		
02-Sep-16	64.8	193	357	368	315	284	210.6	71	32.5	47.0	29.0	7.0	41.0	9.02	5.74	5.48	5.92	6.25	6.31
03-Sep-16	15.0																		
04-Sep-16	11.8																		
05-Sep-16	10.7																		
06-Sep-16	10.0	191	333	606	369	403	550.8	68	29.0	40.0	22.0	10.0	26.0	9.34	5.85	6.31	6.6	7.12	6.95
07-Sep-16	31.8																		
08-Sep-16	67.7																		
09-Sep-16	24.4	171	289	400	349	373	320.7	63	36.0	39.0	31.0	12.0	38.0	8.37	5.85	5.46	5.91	6.21	5.94
10-Sep-16	16.7																		
11-Sep-16	14.0																		
12-Sep-16	10.7																		
13-Sep-16	9.9	190	328	599	344	379	501.7	65	28.0	42.0	26.0	6.0	28.0	8.98	6.06	5.68	6.49	7.09	6.62
14-Sep-16	9.6																		
15-Sep-16	8.3																		
16-Sep-16	7.8																		
17-Sep-16	7.8																		
18-Sep-16	7.5																		
19-Sep-16	26.5																		
20-Sep-16	21.8	190	372	451	397	372	486.2	61	31.0	41.0	26.0	7.5	31.0	7.33	5.22	5.67	5.67	5.86	6.21
07-Feb-17		198	408	727	437	472	592.1	50	34.0	53.0	24.0	10.0	39.0	12.12	12.24	12.8	11.74	12.25	12.31

Table G3. Raw Data (Turbidity, Total Coliforms, E. coli)

	Avg Discharge	Turbidity (NTU) by site						Total Coliform (CFU/100 ml) by site						E. coli Level (CFU/100 ml) by site					
	(ft^3/sec)	A	B	C	D	E	F	A	B	C	D	E	F	A	B	C	D	E	F
16-Jul-16	48.0																		
17-Jul-16	30.5																		
18-Jul-16	36.8																		
19-Jul-16	93.7																		
20-Jul-16	209.9	7.54	9.77	8.28	15.4	16.6	51.1	16640	36540	173290	77010	155310	242000	770.1	1300	2420	1733	3730	9080
21-Jul-16	28.4																		
22-Jul-16	21.6	6.86	4.07	3.05	4.65	2.77	4.38	22820	8620	24890	17890	29090	15390	200	50	630	410	200	310
23-Jul-16	19.8																		
24-Jul-16	17.8																		
25-Jul-16	19.6	6.55	3.61	0.87	3.08	41.9	4	29090	7710	27550	8820	141360	10760	2380	310	520	310	4110	300
26-Jul-16	16.1																		
27-Jul-16	14.8	5.28	3.13	1.22	3.54	9.78	3.51	24890	6090	12460	5710	9330	10140	50	410	200	200	310	310
28-Jul-16	142.5																		
29-Jul-16	458.1	58.5	36.3	11.3	94.2	126	242	344800	517200	579400	461100	686700	816400	17850	7710	14010	14670	34480	21050
30-Jul-16	222.1																		
31-Jul-16	364.0																		
01-Aug-16	141.9	8.61	20.4	2.88	25.3	27.1	30.1	46110	34480	20640	27230	36540	51720	620	520	960	840	970	630
02-Aug-16	99.7																		
03-Aug-16	70.3	8.27	8.66	1.56	11.4	10.5	10.8	51720	8550	21430	13760	12360	17260	410	50	300	410	50	300
04-Aug-16	44.2																		
05-Aug-16	33.8	5.49	4.25	1.46	4.07	3.49	5.34	30760	36540	17850	14210	13140	15150	750	100	100	200	200	200
06-Aug-16	32.5																		
07-Aug-16	27.2																		
08-Aug-16	21.4	5.23	4.63	1.05	3.91	2.23	4.92	21430	32550	18500	20140	7980	5040	410	50	750	200	200	50
09-Aug-16	20.6																		
10-Aug-16	19.3	7.29	4.15	1.51	3.37	2.08	3.59	18600	24810	17850	9330	8840	6270	435.2	117.8	387.3	193.5	143.9	261.3
11-Aug-16	18.6																		
12-Aug-16	16.9	6.45	4.1	3.25	3.36	2.26	2.88	38730	61310	22820	13330	9590	7940	866.4	71.2	344.8	579.4	222.4	613.1
13-Aug-16	20.2																		
14-Aug-16	16.6																		
15-Aug-16	80.9	6.04	3.3	3.4	4.81	3.86	4.28	30760	43520	242000	242000	242000	16580	547.5	344.8	19560	24890	21430	1340
16-Aug-16	226.5																		
17-Aug-16	131.2	10.9	26	2.93	31.5	30.9	28.2	92080	38730	43520	86640	64880	120330	2130	860	1600	1080	1730	1210
18-Aug-16	196.8																		
19-Aug-16	83.5	10.4	12.4	2.3	13.7	17.4	13.2	54750	10120	27550	29090	32550	30760	520	200	520	200	520	860
20-Aug-16	50.8																		
21-Aug-16	186.7																		
22-Aug-16	80.0																		
23-Aug-16	32.5	4.24	4.6	1.5	3.74	2.95	5.84	32550	12960	61310	24810	18500	20140	310	50	520	50	200	630
24-Aug-16	25.3																		
25-Aug-16	21.4																		
26-Aug-16	20.6																		
27-Aug-16	18.1																		
28-Aug-16	15.6																		
29-Aug-16	14.7																		
30-Aug-16	13.9	8	2.84	2.36	2.32	2.51	3.17	43520	8390	173290	14140	8360	7430	435.2	73.3	344.8	290.9	193.5	547.5
31-Aug-16	12.9																		
01-Sep-16	45.7																		
02-Sep-16	64.8	37.5	3.41	3.44	4.58	7.95	27.8	54750	11370	214200	198630	173290	396800	630	100	14390	2920	3990	11120
03-Sep-16	15.0																		
04-Sep-16	11.8																		
05-Sep-16	10.7																		
06-Sep-16	10.0	15.5	2.92	3.49	3.23	1.65	3.11	77010	2419.6	21430	9340	11620	13740	980.4	98.5	410.6	307.6	209.8	435.2
07-Sep-16	31.8																		
08-Sep-16	67.7																		
09-Sep-16	24.4	7.06	5.8	1.71	7.05	5.11	7.26	43520	27230	38730	57940	77010	27550	1350	1480	630	3790	2530	980
10-Sep-16	16.7																		
11-Sep-16	14.0																		
12-Sep-16	10.7																		
13-Sep-16	9.9	6.39	2.67	1.99	3.57	5.82	3.53	36540	5980	24810	6910	11300	6130	307.6	110.6	249.5	344.8	124.6	344.8
14-Sep-16	9.6																		
15-Sep-16	8.3																		
16-Sep-16	7.8																		
17-Sep-16	7.8																		
18-Sep-16	7.5																		
19-Sep-16	26.5																		
20-Sep-16	21.8	7.25	2.5	3.7	3.22	4.91	6.03	23590	19180	141360	29870	141360	111990	860	200	4570	1070	4570	2750
07-Feb-17		2.19	7.88	2.1	8.07	7.49	5.12	866.4	325.5	727	146.4	488.4	1732.9	24.6	20.1	77.1	95.9	70.8	53